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Frank C. Nichols

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ARACHIDONIC ACID METABOLISM IN ROS 17/2·8 CELLS TREATED WITH PARATHYROID HORMONE AND IN HUMAN PERIPHERAL BLOOD MONOCYTES TREATED WITH THE COMPLEMENT FRAGMENT, C3b, AND BACTERIAL LIPOPOLYSACCHARIDE

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Ph.D., University of Rochester, 1982

A Thesis

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ARACHIDONIC ACID METABOLISM IN ROS 17/2•8 CELLS TREATED WITH PARATHYROID HORMONE AND IN HUMAN PERIPHERAL BLOOD MONOCYTES TREATED WITHTHE COMPLEMENT FRAGMENT, C3b, AND BACTERIAL LIPOPOLYSACCHARIDE

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1985

To Nancy

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Introduction:

The polyunsaturated fatty acid, arachidonic acid (AA), can be converted through cyclo-oxygenase and/or lipoxygenase catalyzed reactions to form prostaglandins, thromboxanes, leukotrienes (62), and other products (for review see 76). However, AA must be available to these enzyme systems as a free fatty acid in order for these metabolic reactions to take place (48,101). Free AA levels are normally held to very low concentrations within cells (39,47) and yet AA may comprise a substantial proportion of the constituent fatty acid in cellular lipid pools. Generally, AA is esterified with complex lipids including cholesteryl esters, triglycerides, and phospholipids. Release of AA from complex lipids is therefore a physiological prerequisite for the production of eicosanoid metabolites. While the importance of prostanoids in cell and tissue physiology has been recognized for several decades, it is only recently that considerable progress has been made in the understanding of mechanisms regulating AA release from cellular lipids, primarily phospholipids.

Unique to the process of AA metabolism is the capacity for cells to selectively release AA from phospholipid stores following membrane perturbation with a variety of agents including hormonal, chemical, and mechanical stimuli. In addition, increased AA metabolism is frequently accompanied by rapid compositional changes in membrane phospholipids,

alterations in membrane fluidity, increased levels of cell associated Ca^{++} levels, and alterations in enzyme activity (68). The interdependence of these events in regulating AA metabolism is, as yet, not clearly defined. In addition, the ubiquity of increased AA metabolism following membrane perturbation is by no means established throughout the spectrum of cell types existing in nature.

The purpose of this investigation was to examine AA metabolism in two cell types which have not been previously characterized in this respect. AA metabolism was first examined in the osteoblast-like clonal cell line, ROS 17/2·8, following stimulation with parathyroid hormone (PTH). ROS 17/2·8 cells have been shown to release physiologically significant amounts of the AA metabolite, prostaglandin E_2 (PGE_2), in culture (71) . In addition, these cells demonstrate membrane receptors for PTH and several biological responses to PTH thought to be characteristic of osteoblasts, i.e., increased cyclic-AMP (cAMP) levels and decreased alkaline phosphatase activity (54). AA metabolism was also examined in human mononuclear phagocytes (HMP) following stimulation with the complement fragment, C3b, or lipopolysacchide (LPS). C3b and LPS have been shown to stimulate immunoreactive prostaglandin E (PGE) and thromboxane B_2 (TXB_2) release from freshly isolated HMP in culture (72,80). Prostaglandin release from stimulated monocytes and/or macrophages may be important in

the pathogenesis of chronic inflammatory diseases including periodontal disease.

Literature Review:

Arachidonic acid serves as the substrate for the synthesis of prostaglandins (PG), thromboxanes, prostacyclins, leukotrienes (LT), and other products. The end products of AA metabolism are not stored in cells or tissues but are rapidly synthesized and released following appropriate cell stimulation (42,91). With only a few notable exceptions, the biological effects of eicosanoid metabolites are thought to occur locally in the area of release as these compounds are generally very labile. The importance of PGs and LTs in cell physiology is evidenced by the multitude of cell and tissue effects manifested by the various eicosanoid products. PGs have been shown to play important regulatory roles in inflammatory reactions (99), digestive function (69), cardiovascular and renal physiology (50), reproduction (27), systemic homeostasis (41), and platelet aggregation (59). Thromboxane and prostacyclin are also very important in systemic homeostasis and platelet function (2). The recently discovered leukotrienes have not been extensively characterized with respect to cell and tissue effects with one notable exception. Slow reacting substance of anaphylaxis (SRS-A), identifiable for several decades only as an activity, has recently been shown to be a mixture of leukotrienes C_4 and D_4 (3). While AA metabolite production has been recognized as an important mechanism for modulating cell and tissue function, there are many cell

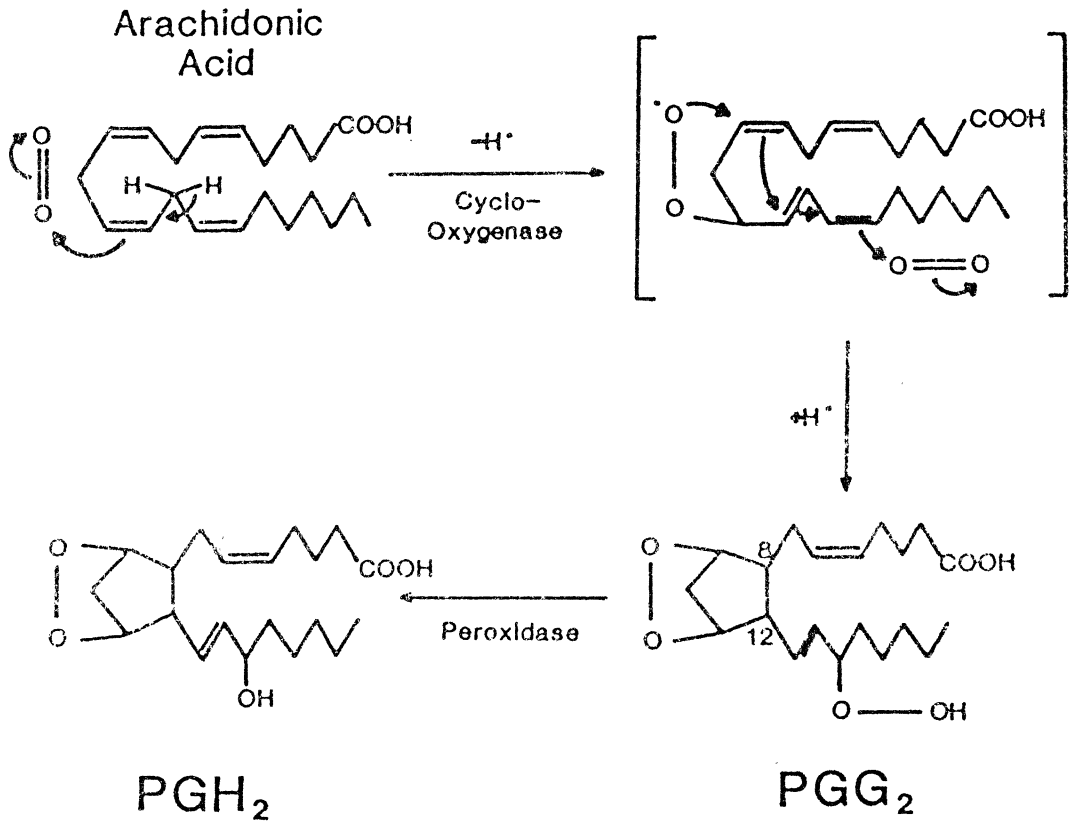
types about which very little is known with respect to AA metabolite release.

Arachidonic acid metabolism to eicosanoid products

Although AA can undergo nonenzymatic conversion through autooxidation to various hydroperoxides, this discussion will be limited to a general description of biologically mediated conversion of AA to its principal metabolites. AA metabolism involves a series of enzyme cascade systems which are preceeded by either an endoperoxidase (cyclo-oxygenase) or lipoxygenase reaction resulting in the production of prostaglandins or leukotrienes, respectively. The principal steps in the endoperoxidase reaction are depicted in Figure 1 (for review see 76). The hallmark of this process is the formation of the cyclopentane ring with the closure of carbons 8 and 12. This basic structure is subsequently modified through additional enzymatic or nonenzymatic reactions to form the various prostaglandins, prostacyclin, HHT, or thromboxanes, as seen in Figure 2. The lipoxygenase pathway first leads to the formation of 5 HPETE (see Figure 3.) which is subsequently converted either to leukotriene A_4 (LTA_4). This unstable intermediate is then converted either to LTC_4, containing a thioether linkage with glutathione in the carbon 6 position, or to LTB_4. Further enzymatic modification of the glutathione residue leads to the formation of LTD_4, LTE_4, and LTF_4.

The production of the previously described metabolites

Figure 1. Reaction scheme for for the conversion of arachidonic acid to PGG_2 and PGH_2. The prominent feature of this reaction is the formation of the cyclopentane ring with closure of carbons 8 and 12.


Arachidonic
Acid
COOH
H
H
O
O
-H·
Cyclo-
Oxygenase
COOH
O
O
O
O
+H·
COOH
OH
Peroxidase
8
12
COOH
O
O
O
OH

PGH_2 PGG_2

Figure 2. Selected metabolic products of PGG_2. In addition to the depicted metabolites, there are several other metabolites known to be produced from PGG_2.

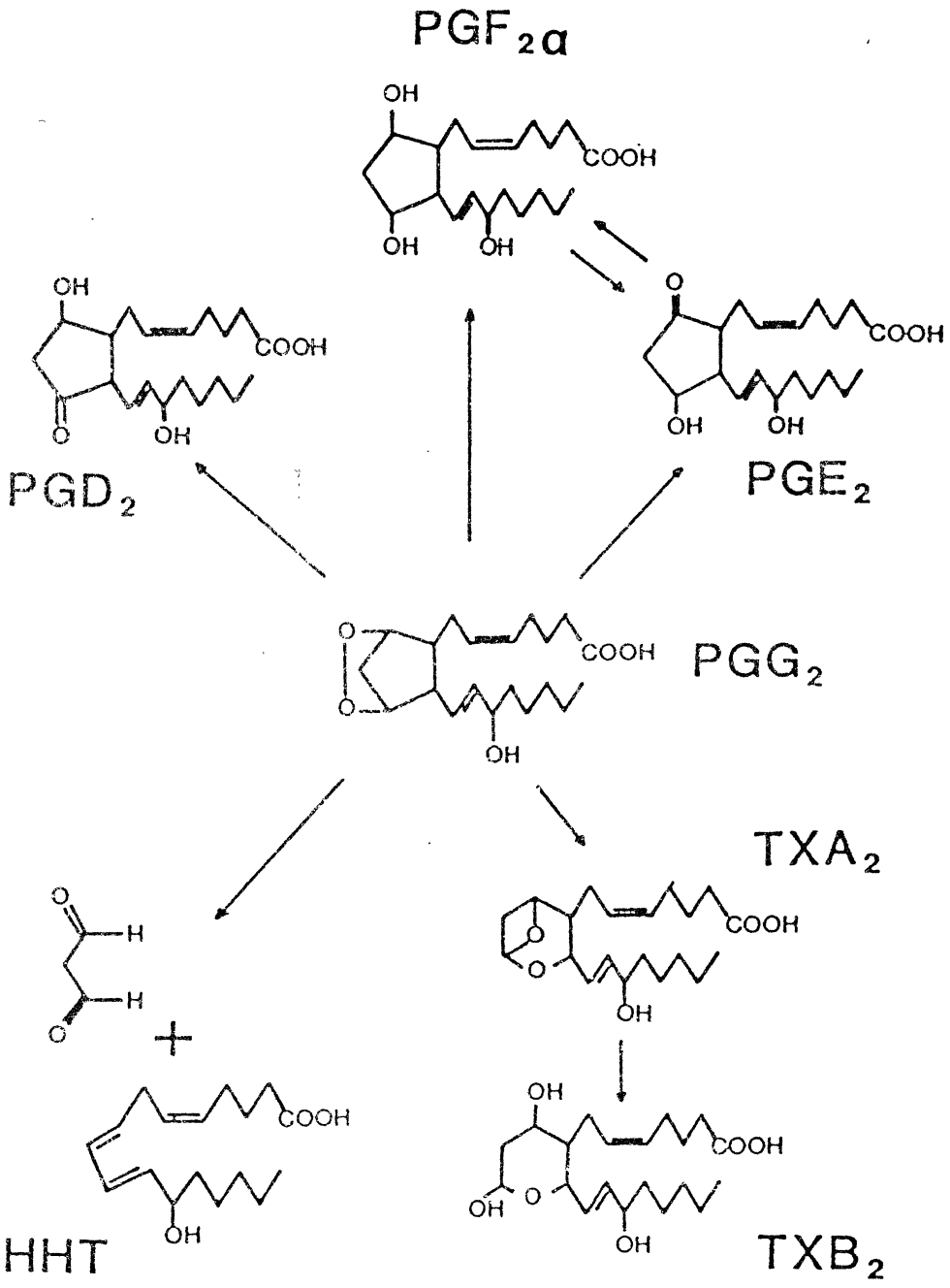
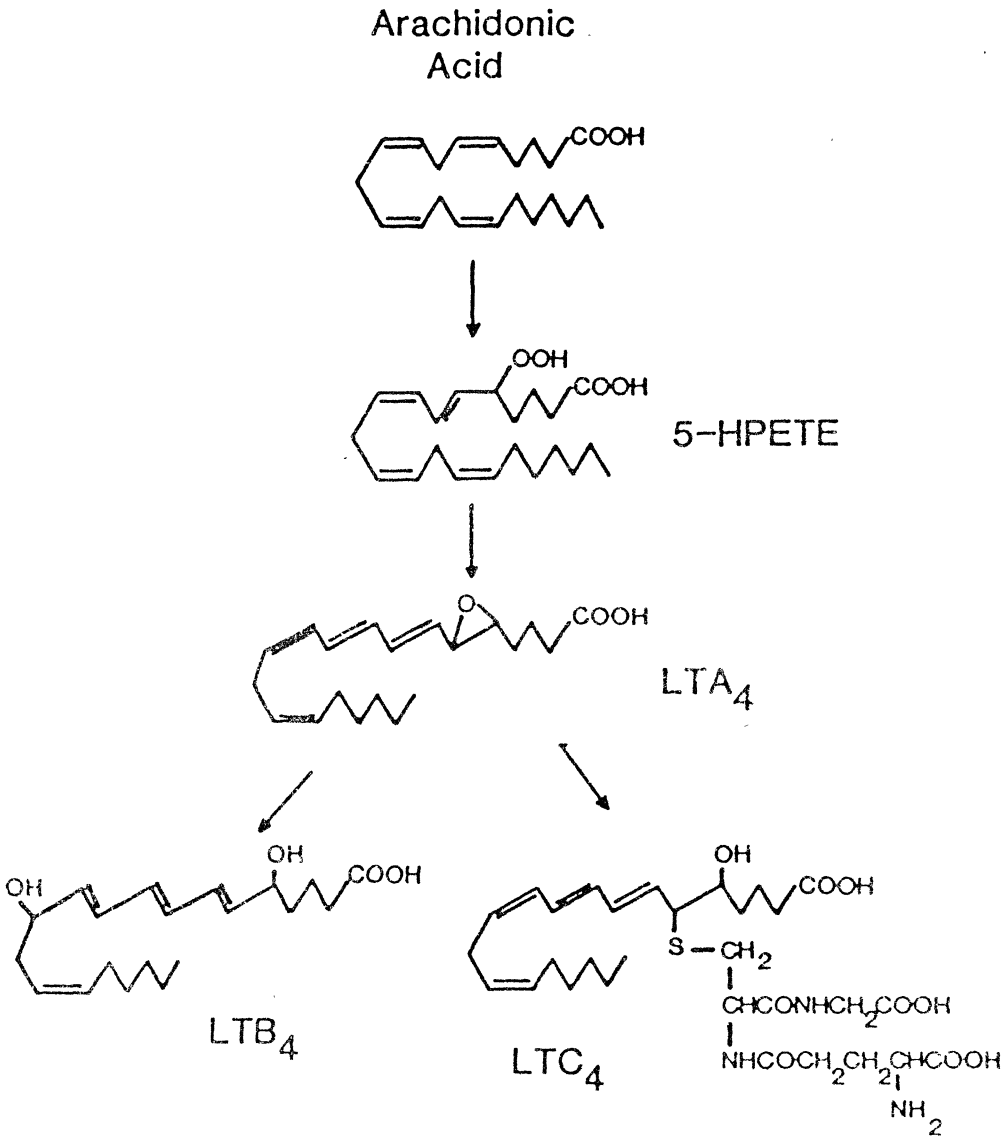

PGF2α
OH
COOH
OH
OH
O
COOH
OH
OH
OH
COOH
O
OH
PGD2
PGE2
O
COOH
O
OH
PGG2
TXA2
O
H
H
O
COOH
O
O
OH
+
COOH
OH
OH
COOH
OH
O
OH
HHT
TXB2

Figure 3. Conversion of arachidonic acid to leukotriene B_4 (LTB_4) or leukotriene C_4 (LTC_4). Further alterations in the glutathione residue of LTC_4 lead to the sequential formation of LTD_4, LTE_4, and LTF_4.


Arachidonic Acid
COOH
OOH
COOH
5-HPETE
O
COOH
LTA4
OH
COOH
OH
LTB4
OH
COOH
S—CH2
CHCONHCH2COOH
NHCOCH2CH2CHCOOH
NH2
LTC4

is ultimately dependent on the availability of free AA for enzymatic conversion. It has also been a general assumption in the prostaglandin field that the rate limiting step in the production of eicosanoids is the rate of release of AA from lipid stores within cells (97). The release of esterified AA from lipid sources is regulated by several metabolic pathways. However, interest has recently focused on phospholipid metabolic pathways which lead to release of AA following stimulation of target cells with hormonal or chemical agents. Increased phospholipid metabolism is associated with preferential release of AA without comparable release of other constituent fatty acids (36,9). Preferential release of AA can be attributed in part to the positional specificity of AA incorporation into phospholipids. AA is esterified predominantly on the number 2 glycerol carbon of phospholipids. Generally, saturated fatty acids are localized to the number 1 carbon of the glycerol moiety of phospholipids whereas the number 2 position is usually occupied by unsaturated fatty acid moieties (96). In addition, many cell types demonstrate enrichment for AA in particular phospholipid classes, most frequently in phosphatidylcholine (PC) and phosphatidylinositol (PI) (1). Although the mechanisms regulating enrichment for AA in certain phospholipids are poorly characterized, those phospholipids which are principal stores for AA are frequently involved in metabolic

processes which lead to selective release of AA. Several pathways have been described to date:

1) Sequential methylation of phosphatidylethanolamine (PE) to form PC followed by release of AA from PC through the action of a PC-specific phospholipase A_2.

2) PI breakdown to diglyceride (DG) with subsequent release of AA from DG through the action of diglyceride lipase.

3) Breakdown of PI to DG followed by conversion of DG to phosphatidic acid (PA). AA is then released from PA through the action of a PA specific phospholipase A_2.

Phospholipid methylation and AA release.

Two principal pathways exist in cells for the synthesis of PC: 1) transfer of phosphocholine from CDP-choline to 1,2 diacylglycerol catalyzed by CTP: phosphocholine cytidyl transferase (81) and 2) three sequential methylations of the amine residue of PE through the action of methyltransferase enzymes (12,26). The later pathway has been shown to accelerate following receptor-ligand interaction in a variety of cell types (31). The methylation process has also been associated with flipping of the monomethyl-PE intermediate across lipid bilayers as there is an asymmetric distribution of methyltransferase enzymes (31). The sequence of events is as follows: on the cytoplasmic side of the plasma membrane, PE undergoes a single methylation through the action of methyltransferase I and is flipped to the exterior side of the plasma membrane. The monomethyl-PE then

undergoes two additional methylations through the action of methyltransferase II to yield PC. Stimulation of this process is associated with alterations in membrane fluidity (32), receptor expression (88), Ca dependent processes (40), and biological responses such as histamine release from mast cells, T lymphocyte blastogenesis, chemotaxis, and cell differentiation (31). This process is also associated with increased cellular lysophosphatidylcholine (lysoPC) levels and enhanced release of AA both resulting from increased phospholipase A_2 action on PC (31). In addition, treatment of cells with the methyltransferase inhibitor, 3-DZA (3), 1) blocks methylation of PE, 2) inhibits biological effects such as histamine release, and 3) prevents the stimulated release of preincorporated radiolabelled AA (31). Although there is a close association between AA release and increased phospholipid methylation in certain cell types, there is no direct evidence which establishes PC as the single source of released AA.

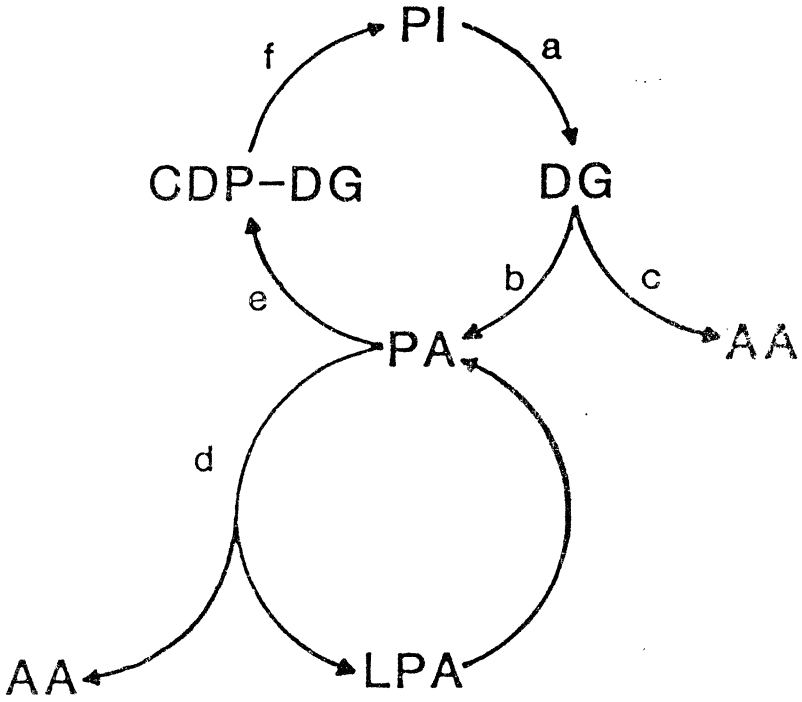
PI-PA cycle and release of AA.

In 1953, Hokin and Hokin noted that acetylcholine stimulated rapid uptake of $^{32}PO_4$ into PI in exocrine pancreas (33). This finding led to the proposal that phospholipid metabolism might be intimately related to the action of this neurotransmitter in pancreatic cells. The effect observed by the Hokins described a small aspect of what is now called the PI-PA cycle. According to current

evidence, hormonal or chemical stimulation of certain cells is associated with rapid degradation of PI through the action of a PI specific phospholipase C (7). This enzymatic breakdown yields 1,2-diacylglycerol (DG) and phosphoinositide. DG may then undergo phosphorylation through the action of diglyceride kinase to produce PA or may be cleaved to monoglyceride and AA through the action of diglyceride lipase (6). PA can then be reconverted to PI through the action of two enzymes, CDP-phosphatidate cytidyl transferase and CDP 1,2-diacylglycerol-inositol phosphatidyltransferase. The salient features of this pathway are depicted in Figure 4.

Stimulated phospholipid labelling within the PI-PA cycle has been described for platelets (7), neutrophils (25), macrophages (34), renal tissues (15), neural tissues (22), and others (33,67), following treatment with a variety of hormones or chemicals. As was described earlier, stimulation of phospholipid turnover in the PI-PA cycle can be associated with increased release of AA either from DG through DG lipase (6) or from PA through a PA specific phospholipase A_2 (8). In addition to increasing AA release, the PA-PI cycle may contribute to cell stimulation through several other mechanisms. DG has been shown to stimulate protein kinase C activity (42,92). Also, lysophosphatidic acid has been proposed to act as a Ca ionophore in cell membranes (49) and has been shown to promote Ca permeability

Figure 4. Phospholipid turnover in the phosphatidylinositol-phosphatidate cycle. Phosphatidylinositol is rapidly broken down to diglyceride (DG) and inositide through the action of PI specific phospholipase C (a). DG is then either deacylated through DG lipase (c) to yield monoglyceride and arachidonic acid (AA) or is phosphorylated through DG kinase (B) to produce phosphatidic acid (PA). Phosphatidic acid may then be deacylated through the action of phospholipase A_2 (d) to yield AA and lysophosphatidic acid (LPA) or can be converted to PI through the action of CDP-phosphatidate cytidyl transferase (e) and CDP 1,2-diacylglycerol-inositol phosphatidyltransferase.


PI
a
f
CDP-DG
DG
b
c
e
PA
AA
d
AA
LPA

in artificial lipid bilayers (84). In summary, increased phospholipid turnover in the PA-PI cycle has been associated with and/or may elicit a variety of molecular signals in stimulated cells including increased release of AA.

Recently, rapid breakdown of another group of phospholipids, the polyphosphoinositols, has been observed following hormonal or chemical stimulation of several cell types (4,30,60). Although the breakdown of these phospholipids has not been directly linked to a stimulation of AA release, they appear to be of sufficient importance to the previously described phospholipid processes to be mentioned here. Polyphosphoinositols include phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-diphosphoinositol (PIP) which constitute quantitatively minor phospholipids in biological membranes. Following stimulation of certain cells with hormones or neurotransmitters, PIP and PIP_2 are rapidly hydrolyzed by phospholipase C to form inositol bisphosphate and inositol trisphophate, respectively, as well as DG (4). This process is similar although more rapid in onset than the previously described breakdown of PI in the PI-PA cycle (4). Inositol triphosphate then stimulates the release of Ca from internal cellular stores (56) whereas DG stimulates protein kinase C activity as described previously (42,92). Ca release from internal stores is thought to precede the influx of Ca from extracellular medium which in many cases

is associated with the previously described alterations in phospholipid metabolism and increased release of AA. However, there is no evidence to date suggesting a cause and effect relationship between PIP and PIP_2 breakdown and Ca influx into cells. On the other hand, the interrelationships between the PI-PA cycle and polyphosphoinositol breakdown together with the relationship between inositol trisphosphate production and Ca release from intracellular stores suggest that these processes are at least indirectly related to stimulation of AA release.

Variation in mechanisms of AA release between cell types.

The previously described phospholipid metabolic pathways are not found in all cell types nor are they the only pathways known to exist. The mechanisms of AA release from phospholipids can vary between tissues, between cell types within the same tissue, and within the same cell type depending on the species and stimulus. For example, AA release from human neutrophils treated with synthetic chemoattractant occurs primarily through phospholipase A_2 cleavage of PI (103). Following stimulation of human platelets with thrombin, AA is released predominantly through the action of phospholipase on PC (104). In peritoneal macrophages stimulated with zymosan, deacylation of PI appears to be the dominant mechanism for the release of AA (20). In contrast, Kroner, et al have shown that free AA levels are primarily regulated by reacylation mechanisms

involving lysophosphatide-acyltransferase catalyzed reactions (45). Finally, there are many cell types which manifest no alterations in phospholipid metabolism and/or AA release when treated with appropriate physiological stimuli (60). In summary, there are many exceptions to the metabolic pathways outlined previously for regulating AA release. Furthermore, there are a great number of cell types which have not been studied with respect to phospholipid metabolism and AA release.

The purpose of this investigation was to study AA metabolism and release from two previously uncharacterized cell types. The first chapter describes experiments examining phospholipid-AA metabolism in the osteoblast-like clonal cell, ROS 17/2·8, following stimulation with PTH. The second chapter describes the partial characterization of AA metabolism in human peripheral blood monocytes <u>in vitro</u> stimulated with the complement fragment, C3b, and lipopolysaccharide.

Chapter 1.

Arachidonic Acid-Phospholipid Metabolism in ROS 17/2·8 Cells Grown in Microcarrier Culture

Abstract:

The present study examined the effect of PTH on arachidonic acid metabolism and release from ROS 17/2·8 cells prelabelled for 24 hr with [^{3}H]AA. ROS 17/2·8 cells were first grown in microcarrier culture to obtain large numbers of substrate attached cells. It was found that PTH (10^{-8}M) slightly stimulated ^{3}H release from [^{3}H]AA prelabelled cells after 12 hr of hormonal stimulation but not after 3 or 24 hr. In contrast, A23187 (5uM), a Ca^{++} ionophore, was a potent stimulator of [^{3}H] release for up to 24 hr. PTH did not alter [^{3}H] label content of ROS 17/2·8 PE, PI, or PC after 24 hr of exposure. Additional experiments examined the effect of PTH on phospholipid [^{32}P]PO_4 labelling in order to assess alterations in phospholipid turnover. PTH was shown to have no significant effect on [^{32}P]PO_4 uptake into PE, PI, PC, and PA in ROS 17/2·8 cells suggesting that phospholipid turnover is not stimulated following hormonal treatment. These results indicate that arachidonic acid and phospholipid metabolism by ROS 17/2·8 cells is not sufficiently affected by PTH to implicate endogenous arachidonic acid release as a mechanism for increased PGE_2 production.

Introduction:

PGE_2 release from rodent osteosarcoma clonal cells has been correlated with the expression of osteoblastic phenotypic characteristics, i.e., decreased alkaline phosphatase activity and increased cAMP levels following parathyroid hormone (PTH) stimulation (70). The cyclooxygenase catalyzed production of prostanoids, including PGE_2, is controlled by the availability of free arachidonic acid (AA) which is normally held to very low levels within cells (100). Two principal mechanisms have been identified whereby cells increase free AA levels: by decreasing the rate of AA acylation into lipid stores within cells (45) and by increasing the rate of AA release from lipid stores (9). Recently, phospholipid turnover has been observed in association with the release of AA from various cell types (29,34,93). Phospholipid turnover events can be initiated by receptor-ligand interaction (22) and can be associated with increases in cellular Ca levels (56), activation of phospholipase C and/or A_2 activity (104), and increased AA release. There is no substantial evidence indicating that receptor-ligand interaction leads to inhibition of AA incorporation into cellular lipids. The release of elevated amounts of PGE_2 by osteoblastic cells in culture indicates that arachidonic acid (AA) is available for conversion to PGE_2. It has also been demonstrated that exogenous AA can be converted to PGE_2 by osteoblast enriched

calvaria cells and osteoblast-like clonal cells (60,70). Recent studies have also shown PTH stimulation of PGE release from osteoblast-enriched cells in culture (53).

The purpose of the this study was to determine whether PTH can stimulate release of arachidonic acid from ROS 17/2·8 osteoblast-like clonal cells in a manner consistent with cell regulated release resulting from increased phospholipid turnover.

Materials and Methods:

Microcarrier cell culture.

ROS 17/2·8 cells were passaged and grown in microcarrier culture (MC). This culture technique was chosen because large numbers of cells are conveniently produced, experiments can be performed without enzymatically releasing cells, and the entire cell culture can be uniformly labelled prior to aliquoting cell samples for experimental use. Briefly, this technique involves growing cells on collagen coated dextran beads (Cytodex III beads, Pharmacia) maintained in suspension with stirring at 30-35 rpm in a microcarrier spinner flask (250 ml, Bellco). Microcarrier beads are 100-150 μm in diameter and provide 0.2-0.3 m^2/g of surface area for substrate attached cell growth. Subculture of ROS 17/2·8 cells was accomplished by aliquoting 30 ml of confluent bead suspension into a sterile bottle. After the beads settled, the medium was decanted and 50 ml of Ca-Mg-free Hank's buffer containing 0.01% trypsin (Sigma) was

added to the settled beads. After standing for 5 min at 37°C, the medium was decanted and a second 50 ml aliquot of trypsin containing buffer was added. The mixture was allowed to stand for 15 to 20 minutes after which most cells were released with gentle swirling. The beads were allowed to settle, the cell suspension decanted, and the ROS cells pelleted and resuspended in F-12 medium. Inoculation of a new culture was carried out by adding 2.0 x 10^7 cells to a spinner flask holding 0.5 g of thrice washed Cytodex III beads in 150 ml of F-12 medium containing 1% kanamycin sulfate and 5% fetal calf serum (FCS). The bead suspension was then stirred continuously for 7 days at 35-40 rpm at 37°C. At two day intervals, cell feeding was accomplished by allowing the beads to settle followed by medium exchange. Medium was equilibrated with an atmosphere of 5% CO_2 in air. All spinner flasks were wrapped in foil to prevent exposure of the proliferating cells to light.

Labelling and hormonal treatment of ROS 17/2·8 cells.

Isotopic labelling of ROS cells was accomplished in a microcarrier spinner flask modified with an access hole in the polyethylene screw top. This permitted insertion of a 5 ml pipette into the stirring bead suspension for withdrawal of experimental aliquots. Cells were labelled with either 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid (AA; New England Nuclear) and/or [^{32}P]orthophosphoric acid (NEN) according to the conditions described in the results

section. Labelled cell samples were placed into glass tubes and incubated for at least 30 min at 37°C prior to hormonal stimulation. For experiments examining [^{3}H] release from [^{3}H]AA prelabelled cells, 100 µl samples were taken from ROS-conditioned medium at designated time intervals and counted by scintillation spectrometry.

Human 1-34 PTH (generous gift of Dr. H. Keutman) was stored at -80°C as a 10^{-5}M stock solution in 0.1% Na acetate (pH 4.0) with 1 mg/ml BSA (Fraction V, Sigma). For experimental use, this stock solution was dissolved in sufficient F-12 medium to deliver the required dose in a total volume of 20 µl. All control tubes received PTH vehicle and sham manipulations. A-23187 was stored at -20°C as a 20 mM stock solution in absolute ethanol.

Methods for lipid extraction and thin layer chromatography.

For experiments examining PTH effects on lipid labelling, incubations were stopped with the addition of organic solvent to the glass culture tubes holding ROS cells. Lipids were extracted utilizing a modification of the method of Garbus et al. (24). According to this method, each tube containing 1 ml of medium plus beads received 4 ml of chloroform:methanol (1:2) followed by a 10 second sonication. After standing for 2 hr, each tube then received 1.5 ml of chloroform and 1.5 ml of 2M KCl with 0.5M K_2PO_4 (pH 7.4). After vortexing, tubes were centrifuged for 10 minutes at 1000 x g and the aqueous phase discarded. The

lower organic phase was then transferred and dried under a stream of N_2 gas. The remaining cell pellet was then assayed for DNA using a modification of the method of Burton (13). Prior to TLC separation of phospholipids, an internal standard of 5 µg of phosphatidic acid (PA) was added to each lipid sample to facilitate identification of labelled PA on TLC plates.

Unidimensional TLC was carried out on 0.2 µm LHP-K silica gel G plates (10 x 20 cm, Whatman). The following solvent systems were used: solvent system A, chloroform:ethanol:triethanolamine:H_2O (30:34:35:8 v/v) (95) and solvent system B, chloroform: pyridine:formic acid (50:30:7 v/v) (21). Solvent system A was used to separate phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC), whereas solvent system B was used to separate PA and PE from other phospholipids. Individual lipid samples were taken up in 20 µl of chloroform, dried to 5 ul, and spotted on TLC plates. This was followed by 4 washes of 5 ul each, spotting each wash below the previous one. Following the separation of phospholipids, plates were dried and sprayed with 1 mM 2-p-toluidinylnaphthylene 6-sulfonate in 50 mM Tris (pH 7.4) and lipids visualized with UV light. Individual phospholipids were identified by cochromatography with authentic standards. Lipid spots were scrapped and counts determined using scintillation spectroscopy. Two dimensional

chromatography according to the method of Yazihara (107) was used to verify one dimensional TLC results.

PTH stimulation of cyclic-AMP in ROS cells.

PTH stimulation of ROS 17/2·8 cAMP was assayed for samples taken from microcarrier culture at days 2,4,6, and 8 after incculation. After sampling, the microcarrier beads were allowed to settle, the culture medium was aspirated, and the cells labelled for 2 hr in 1 ml of F-12 medium containing 2% FCS, 1% kanamycin sulfate, and 1 μCi/ml [^{3}H]adenine (New England Nuclear). The medium was then removed and the microcarrier pellet washed 3 times with F-12 medium. Cell samples were incubated for 1 hr in 1 ml of F-12 medium containing 2% FCS and 1 mM isobutylmethylxanthine. Samples were then treated with hormone or diluent at the indicated concentrations and incubated for 5 minutes. Reactions were stopped with the addition of 25 μl of 25% TCA. Labelled cAMP was then determined according to the method of Solomon. (85).

PTH inhibition of alkaline phosphatase activity in ROS cells.

Alkaline phosphatase activity was assayed in duplicate control and PTH-treated samples taken from each of 3 microcarrier flasks at the indicated time points. After sampling, the cell medium was supplemented to contain 10^{-9} M PTH or diluent and the ROS 17/2·8 cultures incubated for 48 hr. Alkaline phosphatase was solubilized with the addition

of 1 ml of 0.2% Triton X 100 and vortexing. A 50 µl sample was taken for assay of p-nitrophenylphosphatase activity according to the method of Lowry (52) as modified by Majeska et al. (54). The percent of hormonal inhibition of alkaline phosphatase activity (corrected for DNA) was calculated for each microcarrier flask and summed for all flasks.

Determination of DNA content in ROS cell samples.

DNA was determined by colorometric assay according to a modification of the method of Burton (13). Cell pellets were taken up in 2 ml of 0.5N perchloric acid and incubated for 15 min at 70°C, vortexing frequently. Undissolved material was then centrifuged and the supernatant removed followed by the addition of 1 ml of 0.5N perchloric acid. After a second 15 min hydrolysis at 70°C, the undissolved material was pelleted and the supernatants pooled. Fresh diphenylamine reagent (4 ml) was then added to each of the pooled supernatants and the samples incubated for 18 hr at 37°C. Samples were then read at 600 nm against a standard curve of known DNA content.

All results are expressed as the mean ± standard error. Differences between means were evaluated by the Student's t test, as described in the results section.

Results:

Hormonal responsiveness of ROS cells grown in microcarrier culture

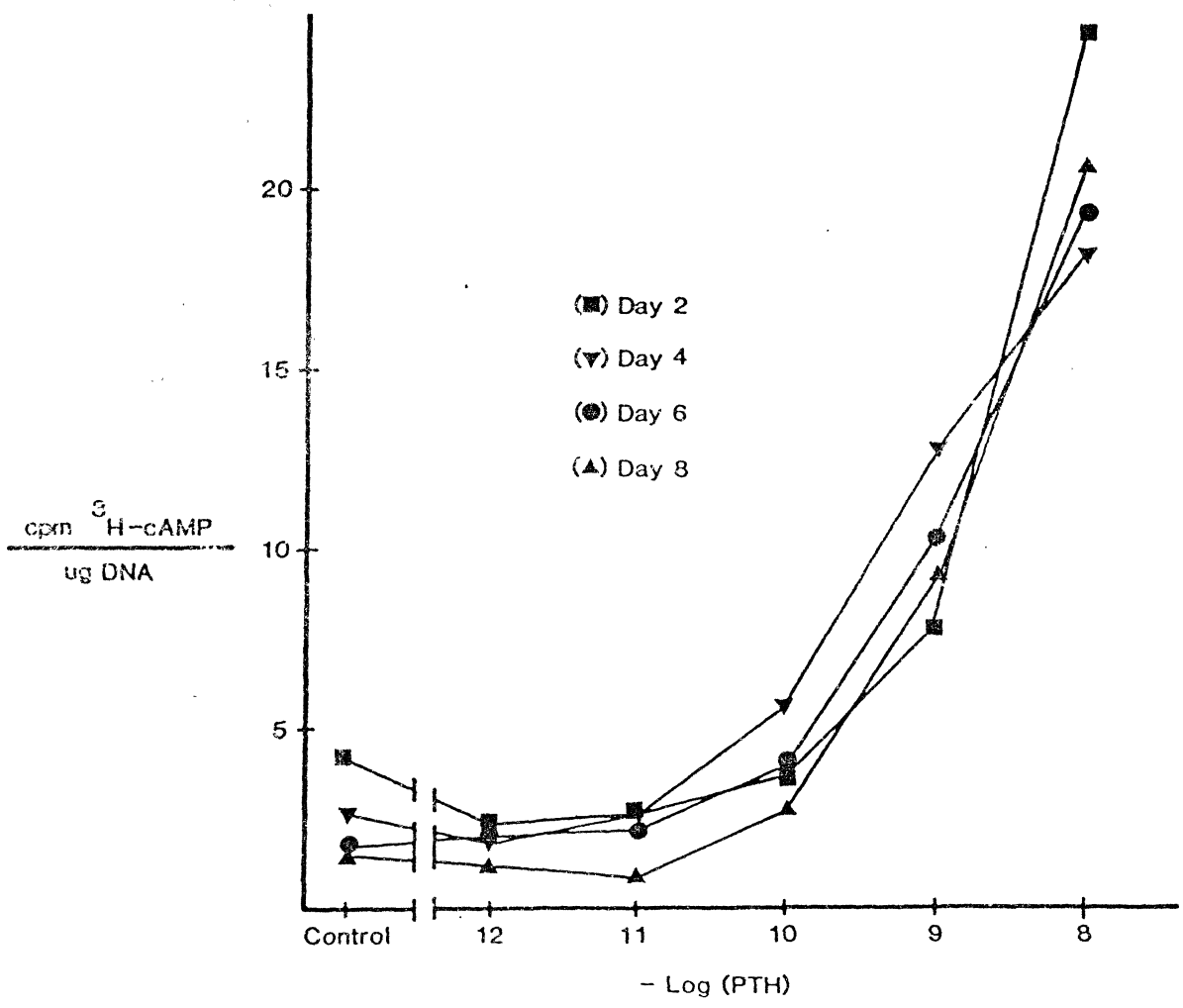
MC has been utilized for the growth and maintenance of

a wide variety of substrate attached cells (16), including bone cells (35). The effect of this culture method on cell properties, such as hormonal responsiveness, has not been established. It was therefore important to compare PTH responsiveness of ROS 17/2·8 cells grown in microcarrier culture with previously characterized responses for cells grown on conventional plasticware. Figure 1 shows the PTH dose dependent stimulation of ROS 17/2·8 cAMP levels with increasing time of growth in MC. There does not appear to be any significant alteration in cAMP response with increasing time in MC. Figure 2 shows that PTH inhibition of ROS alkaline phosphatase was highly dependent on the time in culture. As shown, maximal inhibition of ROS alkaline phosphatase occurs for cells grown for 4-8 days in microcarrier culture. Majeska et al. (54), observed slightly greater inhibition of alkaline phosphatase with 10^{-9}M PTH, assayed on cells grown for 7 days in conventional culture.

[^{3}H]AA labelling of ROS phospholipids in micocarrier culture.

Phospholipid extractions were carried out using a modification of the method of Garbus et al.(24). Using this method, a single chloroform:methanol wash extracted greater than 92 $\pm$ 1.6% (3 trials) of the available [^{3}H] counts in ROS cell samples labelled as previously described. Presumably, all label would not be extracted from these samples, particularly since AA does not efficiently

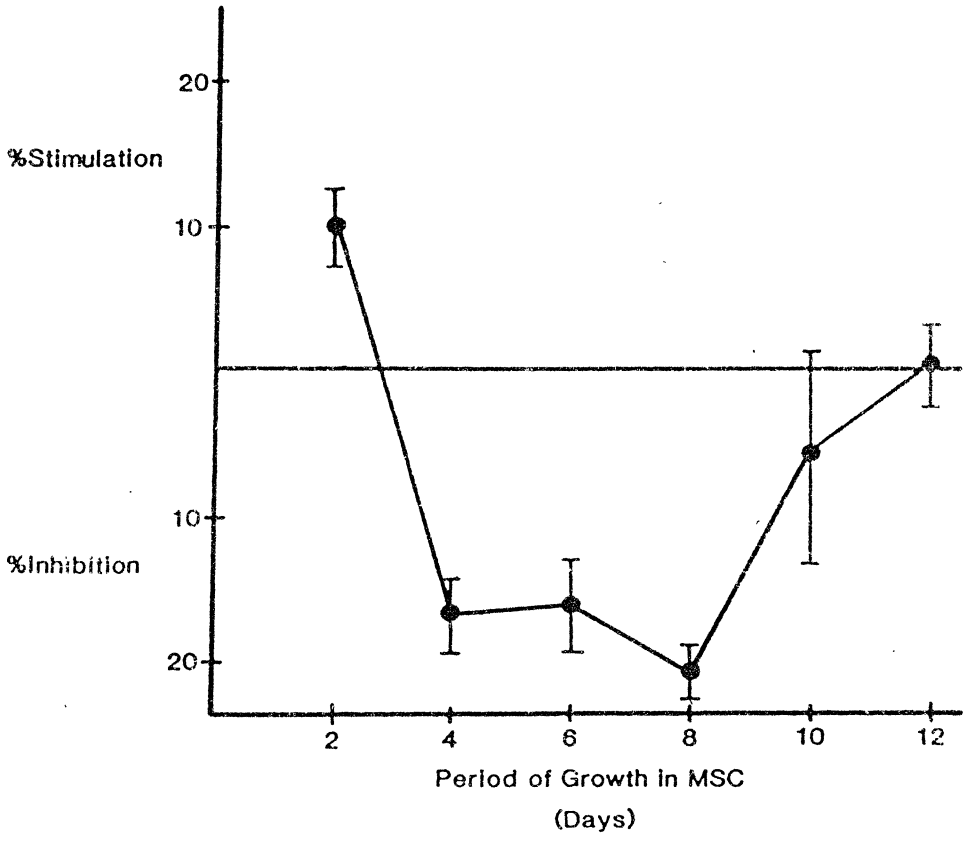
Figure 1. stimulation of ROS 17/2·8 cyclic AMP levels by PTH. Stimulation of cyclic AMP levels was determined for ROS 17/2·8 cells grown for 2(■), 4(▼), 6(●), and 8(▲) days in MC (n=4 for each point).


cpm ^{3}H-cAMP / ug DNA
20
15
10
5
(■) Day 2
(▼) Day 4
(●) Day 6
(▲) Day 8
Control
12
11
10
9
8
– Log (PTH)

partition into organic solvent in the presence of neutral pH-high salt aqueous solvent. Acidic conditions facilitate the extraction of AA as well as acidic phospholipids. An additional chloroform:methanol wash of the cell samples recovered only an additional 3% of the total available counts whereas reextraction with chloroform:methanol:HCl (2:1:0.4) recovered the remaining [^{3}H] counts (~4%). Since the majority of [^{3}H]AA was incorporated into PI, PE, and PC, the recovery of these phospholipids was assumed to be high. However, the recovery of PA was of concern because it is the most acidic of the phospholipids and it is present in very low levels in ROS 17/2·8 cells. Three extraction methods were compared in their efficiency for PA and PE extraction. It was found that a single wash with acidic chloroform:methanol according to the method of Billah et al. (7), recovered significantly less [^{3}H] labelled PE and PA than the method of Garbus et al.(24). In addition, reextraction of the extracted cell samples (Garbus method) with acidic chloroform:methanol did not recover significantly more counts in PE and PA. All lipid extractions in the present study, therefore, utilized the method of Garbus et al. (24).

To examine the stability of [^{3}H]AA labelling of ROS phospholipids in microcarrier culture, cells were prelabelled with [^{3}H]AA for 24 hr and transferred from MC to stationary culture in glass tubes. The time dependence of

Figure 2. Effect of 10^{-9}M PTH on alkaline phosphatase activity in ROS 17/2·8 cells maintained in MC. Cells were cultured for the indicated period in MC and aliquoted into glass tubes. Following stimulation with hormone or diluent, cells were cultured for 48 hr in glass tubes and harvested. Alkaline phosphatase was standardized against cell DNA.


20
%Stimulation
10
10
%Inhibition
20
2
4
6
8
10
12
Period of Growth in MSC
(Days)

label redistribution in ROS phospholipids was determined from the point of sample transfer. Figure 3 shows the distribution of [^{3}H]AA in the major phospholipids of ROS cells following removal from MC. Incubations were terminated with the addition of organic solvent. As shown, there is little redistribution of label following the removal of ROS 17/2·8 cells from MC. Since there was a small decrease in PE labelling for up to 10 min after removing cells from MC, all subsequent experiments allow cultures to equilibrate for at least 30 minutes prior to stimulation with PTH.

PTH effects on [^{3}H]AA release from ROS cells.

Figure 4A demonstrates the extent of stimulation of [^{3}H] label appearance in ROS 17/2·8 cell culture medium following stimulation with PTH or A-23187. Cells were grown for 7 to 14 days in MC, prelabelled with [^{3}H]-AA for 24hr, and washed with unlabelled medium immediately prior to aliquoting cell suspension into glass tubes. [^{3}H] release was significantly increased ($p<0.05$) by 12 hr of PTH treatment whereas 3 and 24 hr of hormone exposure were without significant effect. Figure 4B depicts the release of label from a representative cell preparation. Consistent with the capacity of A-23187 to stimulate Ca dependent phospholipase activity (65), the release of [^{3}H] label from ROS cells was markedly stimulated with A-23187 treatment. The magnitude of the Ca ionophore effect indicates that PTH is not a potent stimulus of this process. The PTH stimulated

Figure 3. Effect of removing ROS 17/2·8 cells from MC on label distribution in major phospholipids. Cells were grown for 7 days in MC and labelled only for the last 24 hr with [^{3}H]AA (1 μCi/100 ml). Aliquots of the cell suspension were transferred to glass tubes at time 0 and organic solvent added at the indicated time points. Each point represents the mean $\pm$ S.E. for 6 trials. Samples contained approximately 1.5 x 10^6 cells / tube as determined by DNA content.

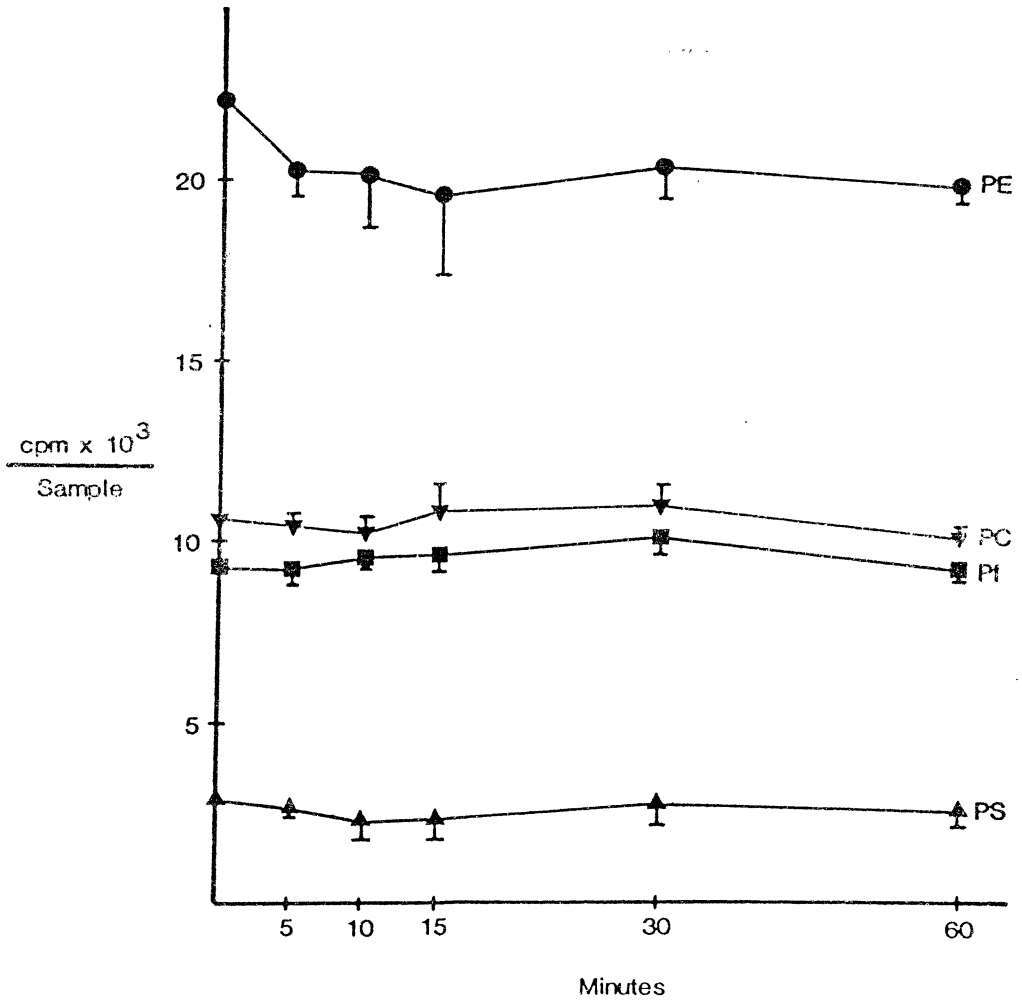
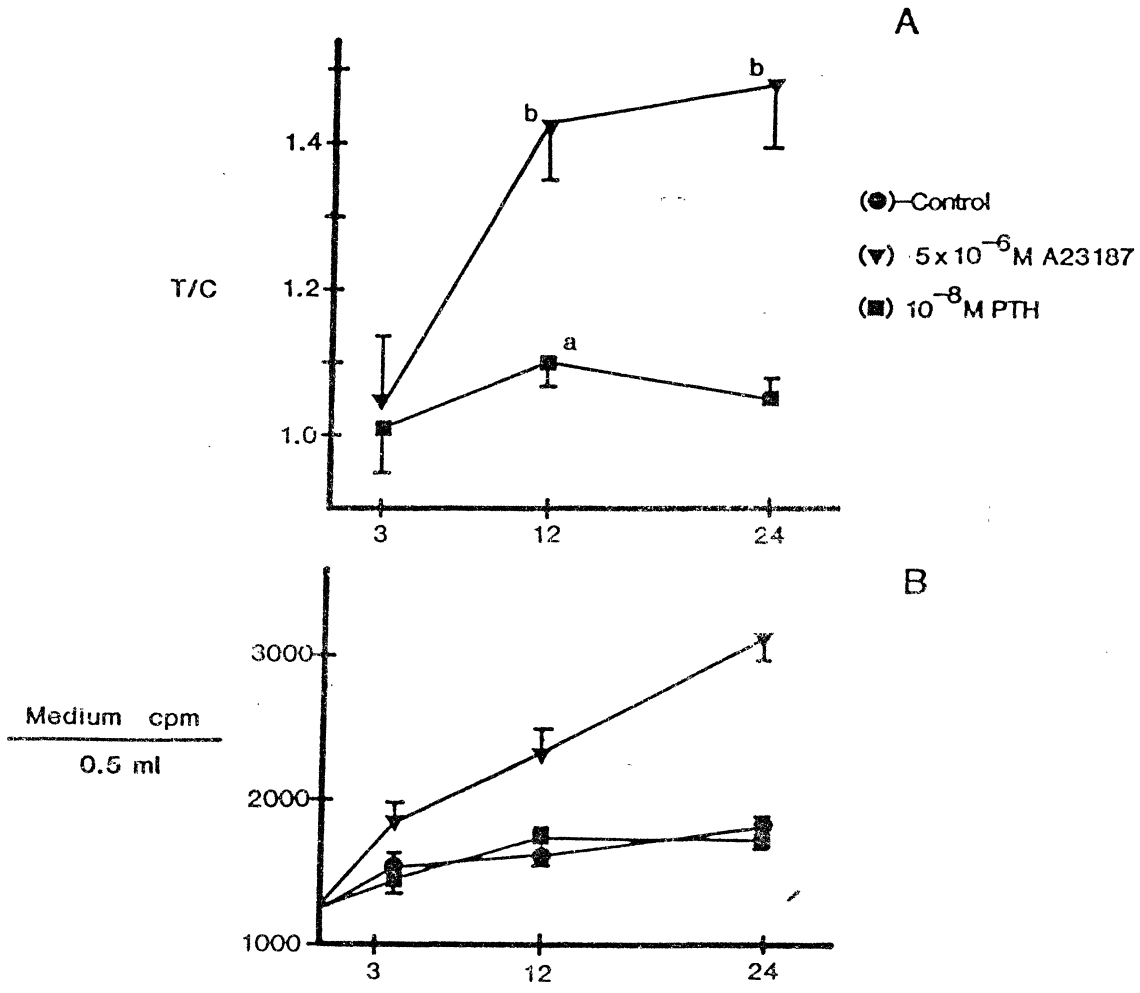

cpm x 10^3
Sample
20
15
10
5
PE
PC
PI
PS
5
10
15
30
60
Minutes

Figure 4. PTH stimulation of [^{3}H] label release from [^{3}H]AA prelabelled ROS 17/2·8 cells. Cells were grown for 7-14 days in MC and labelled for 24 hr prior to dispensing the cell suspension. Cells were washed with unlabelled medium immediately before cell suspension was transferred to glass tubes (5 ml aliquots). [^{3}H] appearance was quantitated by removing 0.5 ml of culture supernatant at the indicated time points. Results are depicted as % stimulation of [^{3}H] release in (A) and a representative release experiment in (B). Results are for 10^{-8} M PTH (■), 5 uM A-23187 (▼), and vehicle control (●). Each point in (a) represents the mean ± S.E. for 7 points. Significant stimulation was observed for (a) 10^{-8}M PTH at 12 hr ($p<0.05$) and (b) A-23187 (10^{-5}M) at 12 and 24 hr ($p<0.005$).


A
T/C
1.4
1.2
1.0
b
b
a
3
12
24
(●)–Control
(▼) 5x10⁻⁶M A23187
(■) 10⁻⁸M PTH
B
Medium cpm
0.5 ml
3000
2000
1000
3
12
24

release in these experiments was observed only when labelled cells were washed with unlabelled medium prior to hormonal treatment. When 24 hr prelabelled cells were stimulated without exchanging medium, PTH had no effect on medium [^{3}H] content (data not shown).

Table 1 shows the distribution of [^{3}H] and [^{32}P] label within ROS 17/2·8 phospholipids in control and PTH treated cultures at 1 min and 24 hr after hormone addition. Cells were prelabelled for 24 hr with either [^{3}H]AA or [^{32}P]PO_4 prior to stimulation. The medium was not exchanged prior to aliquoting cell samples, in an effort to maintain the appropriate steady state for determining PTH effects. PTH at 10^{-8}M or 10^{-10}M had no significant effects on [^{3}H] or [^{32}P] distribution in ROS phospholipids. In addition, 24 hr exposure to PTH had no significant effect on DNA content of ROS 17/2·8 cultures.

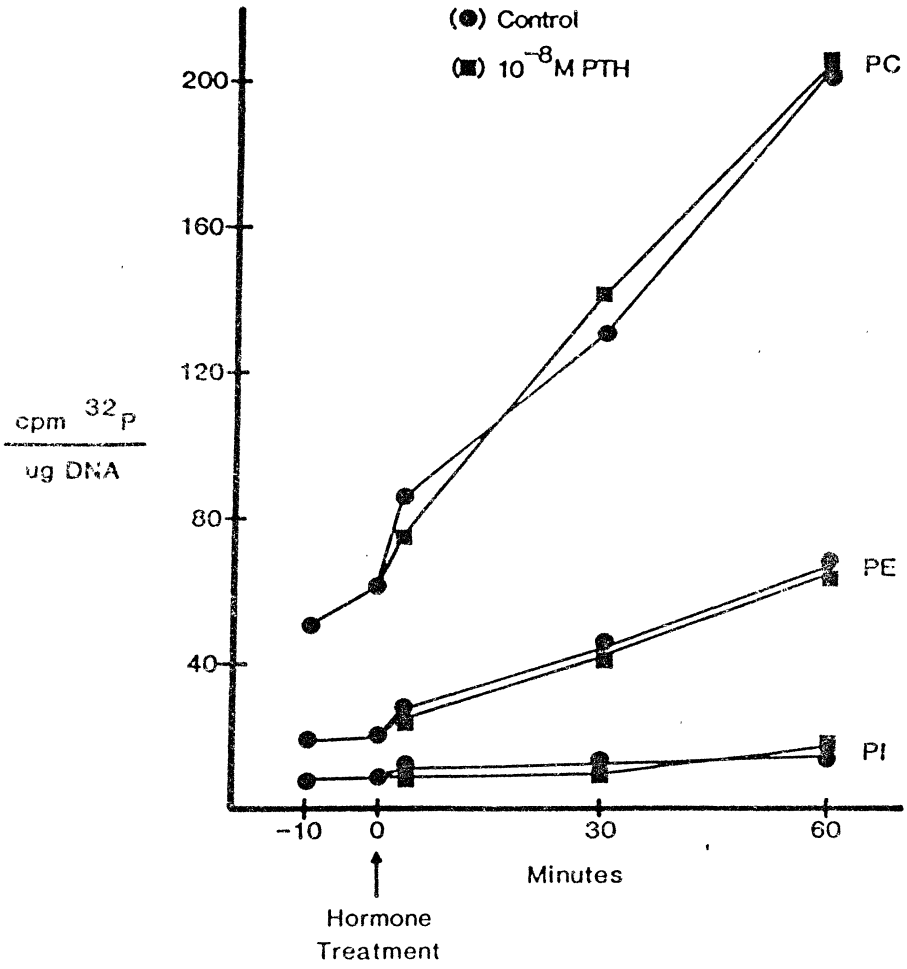
The increased rate of [^{3}H] release observed following PTH treatment of ROS cells might result from a stimulation of phospholipid metabolism without affecting the long term labelling of phospholipids. This process can be monitored by measuring the uptake of label into phospholipid pools following hormonal stimulation. Figure 5 presents the time dependent uptake of [^{32}P]PO_4 into ROS phospholipids with and without PTH treatment. ROS cells maintained in MC were labelled for 2 hr with 100 μCi [^{32}P]PO_4/200 ml of F-12 medium. At that time, aliquots of cell suspension were

Table 1

Phospholipid	CPM/Cell Sample 10^{-8}M PTH	10^{-10}M PTH	Control
^{3}H-PC	5619 ± 353	5750 ± 234	5824 ± 748
^{32}P-PC	17563 ± 1696	15560 ± 657	16533 ± 3112
^{3}H-PI	3892 ± 443	3577 ± 411	3578 ± 354
^{32}P-PI	3577 ± 411	3565 ± 324	3387 ± 323
^{3}H-PE	7838 ± 712	7593 ± 602	7560 ± 765
^{32}P-PE	6569 ± 1069	6450 ± 984	6673 ± 730
DNA (ug/cell sample)	25.6 ± 1.9	23.7 ± 2.3	25.6 ± 1.5

Table 1. Effect of PTH on [^{3}H]AA and [^{32}P]PO_4 content of ROS 17/2·8 phospholipids. Cells were grown for 6 days in MC and labelled for 24 hr with [^{3}H]AA (1 μCi/100ml) and [^{32}P]-PO_4 (50 μCi/200ml). Without exchanging medium, labelled cell suspension was transferred to glass culture tubes and treated with hormone or diluent. ^{3}H counts were corrected for ^{32}P coincidence. All data are expressed as mean ± S.D. for 5 trials.

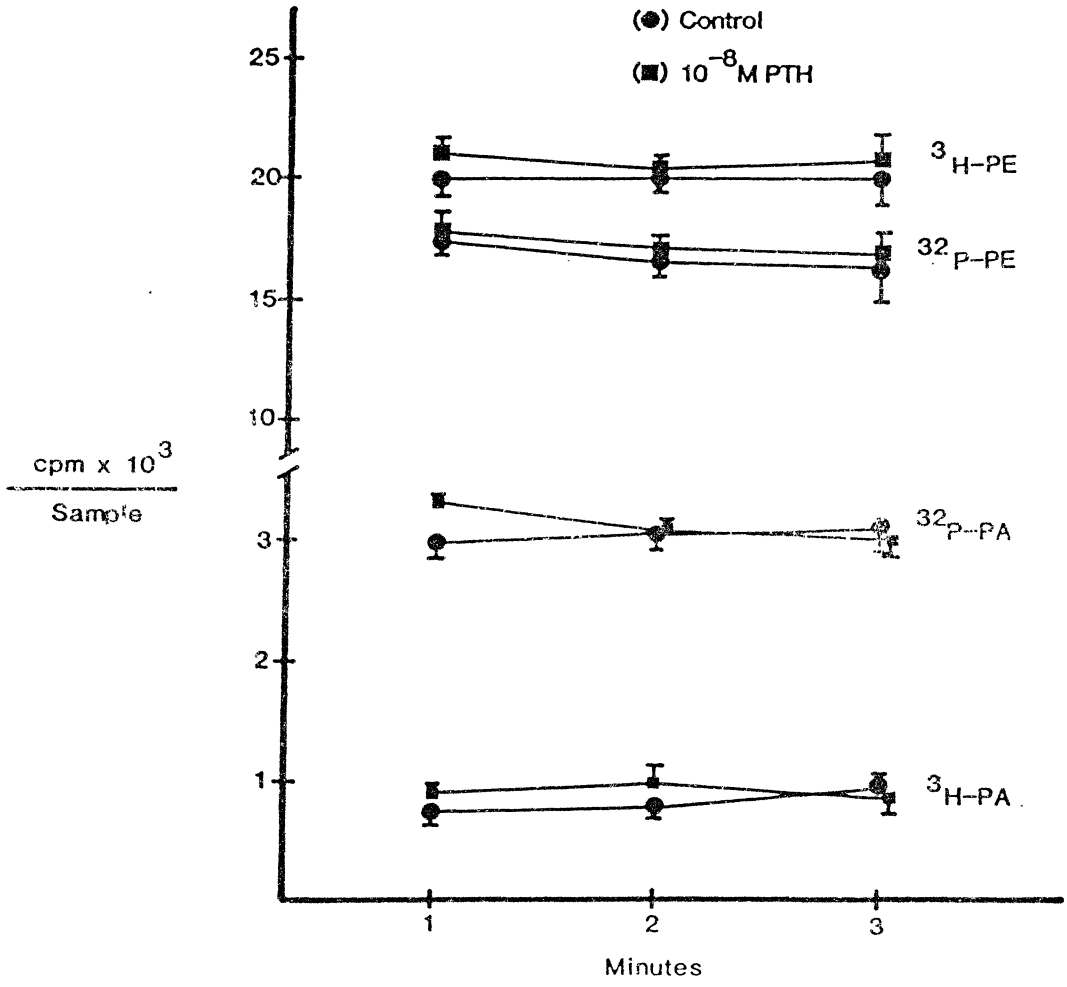
Figure 5. The effect of PTH on [^{32}P]PO_4 uptake into ROS 17/2·8 phospholipids. Cells were grown for 7 days in MC and labelled for 2 hr with [^{32}P]-PO_4 (100 μCi/200ml) prior to transferring cell suspension to glass tubes (1.5 x 10^6 cells/tube). Hormone (10^{-8}M) (■) or diluent (●) was added at 0 min. Each point represents the mean ± S.E. for 3 trials.


(●) Control
(■) 10⁻⁸M PTH
PC
PE
PI
200
160
120
80
40
cpm ³²P / ug DNA
-10
0
30
60
Minutes
Hormone Treatment

placed in glass tubes and incubations were stopped at the indicated time points. As shown, PTH treatment did not significantly alter [^{32}P]PO_4 uptake into PC, PE, and PI during 1 hr of of hormone treatment.

Although it has not been possible to identify PTH induced alterations in [^{3}H]AA content of the major phospholipids in ROS cells, the small increase in [^{3}H] release from PTH-treated ROS cells allows the possibility that label is being released from quantitatively minor lipid classes. Phosphatidic acid (PA) is generally found in very low levels in cells, yet rapid phosphorylation of PA can be observed within seconds to minutes following chemical or hormonal stimulation of certain cell types. The following experiments examined whether PTH could promote PA phosphorylation in ROS cells. Figure 6 shows that PTH had no effect on [^{32}P]PO_4 uptake into PA in ROS 17/2·8 cells nor was there any effect on [^{3}H]AA content in PA. Short term [^{32}P]PO_4 label (2 hr) was utilized to measure PA turnover whereas long term [^{3}H]AA label (24 hr) was thought to reflect alterations in PA arachidonic acid content. PE labelling was included as a reference lipid for PA labelling.

Figure 6. The effect of PTH on [^{32}P]PO_4 uptake and [^{3}H]AA content of phosphatidic acid in ROS 17/2·8 cells. Cells were grown in MC for 6 days followed by 22 hr labelling with [^{3}H]AA (1 μCi/ 100ml). The MC medium was then exchanged with phosphate free Hanks salts and cells labelled for 2 hr with [^{32}P]PO_4 (10 μCi/ 100ml). Suspension aliquots were then transferred to glass tubes (1.5 x 10^6 cells/tube) and cells stimulated with 10^{-8}M PTH (■) or diluent (●). Data points represent the mean ± S.E. for 5 trials. PE was included as a reference lipid.


(●) Control
(■) 10⁻⁸M PTH
25
20
15
10
3
2
1
cpm x 10³
Sample
³H-PE
³²P-PE
³²P-PA
³H-PA
1
2
3
Minutes

Discussion:

Microcarrier culture provides an efficient technique for growing large numbers of substrate attached ROS 17/2·8 cells. In addition, aliquots of confluent bead suspension demonstrated a high degree of uniformity with respect to DNA content and label distribution in phospholipids. In these experiments, it was also important to avoid mechanical harvesting of cells in aqueous medium prior to organic solvent extraction of phospholipids as this manipulation can affect phospholipid metabolism (17). Cells grown on conventional plastic dishes must be mechanically harvested prior to extraction of lipids since organic solvent will dissolve most plastic vessels. By aliquoting cells from microcarrier culture to glass tubes, it was possible to hormonally stimulate cells and extract phospholipids in the same vessel without an intervening mechanical harvesting.

The PTH responsiveness observed for ROS 17/2·8 cells grown in MC was comparable to that previously observed for cells grown on conventional plasticware. The cAMP dose response for ROS 17/2·8 cells grown in MC was virtually identical to that reported for cells grown on plastic (54). Maximal stimulation of cAMP levels with 10^{-8}M PTH was approximately 10 fold (see figure 1), somewhat less than previously reported (54). However, cAMP stimulation was evaluated several times over the course of the current study and maximal stimulation of cAMP with 10^{-8}M PTH was

subsequently found to be approximately 60 fold. The magnitude of the cAMP response, therefore, appears to be dependent on additional factors beyond hormone dose alone.

PTH (10^{-9}M) inhibition of alkaline phosphatase in ROS cells grown for 7 days in MC was also reduced when compared to previously reported responses observed with cells grown for 7 days on plastic (54). However, these cultures were assayed for PTH inhibition of alkaline phosphatase at the same time that reduced cAMP responses were observed. No additional assays were attempted to determine whether PTH inhibition of alkaline phosphatase varied as widely as cAMP responses to a given dose of hormone. To our knowledge, these results are the first to demonstrate hormonal responsiveness of osteoblast-like cells grown in MC.

The appearance of [^{3}H] in medium conditioned by [^{3}H]AA labelled ROS 17/2·8 cells treated with A-23187 reflected the capacity of microcarrier grown cells to release preincorporated arachidonic acid. Relative to the A-23187 effect, PTH had only a slight effect on [^{3}H] release. PTH was found to stimulate the release of [^{3}H] only when labelled cells received fresh medium immediately prior to hormonal stimulation. The increased release of [^{3}H] in these experients presumably represents a more rapid rate of tracer release from PTH treated cells. However, cells which did not receive fresh medium demonstrated no increased release of [^{3}H] label into the surrounding medium after 24 hr PTH

treatment (data not shown). These results suggest that a net release of [^{3}H] label from ROS cells is required to observe PTH effects on label release. Interestingly, [^{3}H] levels in ROS 17/2·8 conditioned medium were identical after 24 hr of culture regardless of whether cells were maintained in MC or in glass tubes (data not shown). Stimulated release of [^{3}H] following treatment with A 23187 indicates that ROS cells possess the capacity to release increased amounts of AA with appropriate stimuli. However, the relatively small increase in [^{3}H] release following PTH treatment suggests that this hormone is not important in this process.

Attempts to identify alterations in [^{3}H]AA content of phospholipids in ROS 17/2·8 cells following PTH treatment yielded negative results. These experiments examined [^{3}H] content of ROS phospholipids for up to 24 hr of PTH exposure. Label incorporation into major phospholipids after 24 hr exposure to [^{3}H]AA was unchanged after an additional 24 hr incubation with 10^{-8}M PTH. These results indicate that PTH does not selectively transfer AA from or between the major phospholipids in ROS 17/2·8 cells. That phospholipid metabolism was not affected by 24 hr exposure to PTH was also supported by experiments utilizing [^{32}P]PO_4 as tracer. ROS 17/2·8 cells were labelled for 24 hr with [^{32}P]PO_4 according to the scheme described for [^{3}H]AA labelling. It was demonstrated that [^{32}P]PO_4 content of major phospholipids in ROS cells remained virtually unchanged for

24 hr control and PTH treated cultures. In summary, PTH treatment was without effect on [^{3}H]AA release from major phospholipids in ROS cells and was unable to alter [^{32}P]PO_4 labelling of phospholipids.

PTH-stimulated release of [^{3}H] label into ROS 17/2·8 conditioned medium might be a manifestation of altered AA metabolism resulting from increased phospholipid turnover. Enhanced AA release has been directly associated with stimulation of phospholipid turnover in a variety of cell types following stimulation with cell specific hormones or certain chemical agents (34,93). Generally, metabolic labelling consistent with increased turnover is observed within seconds to minutes following cell stimulation and is limited to certain phospholipid metabolic pathways. Two major pathways have been identified to date: 1) sequential methylation of PE to PC with subsequent release of fatty acid from PC (31) and 2) rapid breakdown of PI and polyphosphoinositols (DPI and TPI) to diglyceride with subsequent synthesis of PA and/or release of fatty acid from diglyceride (104). In dog kidney slices, [^{32}P]PO_4 incorporation into PI, TPI, DPI, and PA is stimulated within 5 minutes of PTH treatment in a manner consistent with increased phospholipid turnover (58). In the present study, PTH was shown to have no effect on [^{32}P]PO_4 uptake into PI, PC, and PE for up to 60 minutes of hormone exposure. Additional experiments also demonstrated that PA labelling

in ROS 17/2·8 cells was unaffected by PTH treatment. These findings, therefore, indicate that PTH does not stimulate phospholipid turnover in ROS cells in a manner comparable to that previously observed in another PTH target tissue, the kidney.

In conclusion, the results of the present study indicate that PTH does not sufficiently affect the metabolism of [^{3}H]-AA in ROS cells to be considered an important factor in endogenous AA metabolism in this cell type. The apparent capacity of ROS 17/2·8 cells to release significant quantities of PGE_2 may relate to unusually high basal phospholipid turnover (69) although the possibility that exogenous AA is the predominant substrate in PGE_2 production cannot be excluded. In any case, ROS 17/2·8 AA and phospholipid metabolism does not appear to be regulated by PTH such that increased production of PGE_2 would result from release of AA from endogenous esterified AA sources. This does not exclude the possibility that PTH may affect phospholipid metabolism and AA release in non-transformed osteoblasts or in another subgroup of osteoblastic clonal cells.

Chapter 2.

C3b and LPS stimulated release of PGE and TXB_2 from human mononuclear phagocytes in culture: Evidence for independent conversion of arachidonic acid to PGE_2 and TXB_2.

Abstract:

C3b treatment of human peripheral blood mononuclear phagocytes (HMP) in culture stimulates an early release of thromboxane B_2 (TXB_2) and a delayed release of prostaglandin E (PGE) into culture supernatants. We examined C3b stimulated eicosanoid production in ^{3}H-arachidonic acid (AA) prelabelled HMP by comparing the time course of ^{3}H release from HMP phospholipids with the appearance of ^{3}H-labelled PGE_2, TXB_2, and AA in culture supernatants. In addition, we compared the time course of immunoreactive PGE and TXB_2 release with radiolabelled PGE_2 and TXB_2. Identification and quantitation of radiolabelled AA metabolites was performed by TLC and results were corroborated by high pressure liquid chromatography (HPLC). Following C3b treatment of HMP, the time course of radiolabelled TXB_2 release was found to be comparable to TXB_2 measured by radioimmunoassay (RIA). In addition, the stimulated release of ^{3}H label from HMP phospholipids corresponded to the appearance of labelled TXB_2. However, radiolabelled PGE_2 release was maximally stimulated by 8 hr of C3b treatment whereas immunoreactive PGE release was maximally stimulated after 16 hr of treatment. In addition, stimulated release of [^{3}H]AA into culture supernatants was observed prior to any detectable increased release of labelled TXB_2 or PGE_2. The apparent differential release of immunoreactive PGE vs ^{3}H-labelled PGE_2 from C3b treated HMP was further examined by measuring

HMP eicosanoid release following treatment with bacterial lipopolysaccharide (LPS). It was found that LPS did not stimulate labelled PGE_2 release from HMP but significantly stimulated release of immunoprecipitable PGE after 8 hr of treatment. As with C3b stimulation of HMP, the appearance of [^{3}H]AA in HMP culture supernatants did not parallel labelled TXB_2 or PGE_2 release following stimulation with LPS. These results are consistent with independent metabolism of TXB_2 and PGE_2 in stimulated HMP and suggest a complex relationship between AA metabolism and TXB_2 and PGE_2 production in HMP.

Introduction:

Monocytes and macrophages (MP) have been shown to release significant quantities of arachidonic acid (AA) metabolites following stimulation with C3b cleavage fragments (72), antigen-antibody complexes (63), Ca^{++} ionophore, A-23187 (106), concanavalin A (34), LPS (75), zymosan (102), phorbol myristate acetate (37), and other factors (38). To date, the principal metabolites shown to be released by the MP include TXB_2, PGE_2, 6-keto $PGF_{1\alpha}$ (the stable breakdown product of PGI_1), $PGF_{1\alpha}$, leukotrienes (10), and HETES (82). Although there is considerable variation in the production of these eicosanoids from MP preparations (82,23,46), the production and release of AA metabolites is thought to be limited by the availability of free AA to cyclooxygenase and lipoxygenase enzyme systems (86). Since free AA is normally restricted to very low levels within cells, the regulation of AA release from cellular lipid stores appears to determine the rate of AA metabolite production. Two mechanisms have been reported which account for increased free AA levels in stimulated macrophages. These are 1) increased release of AA from phospholipids primarily through the action of phospholipase A_2 (100) and 2) decreased reacylation of AA into lipid pools (45). AA constitutes 20% of the esterified fatty acid in rabbit MP (57) and in human monocytes (87). In addition, labelling experiments demonstrate that AA is selectively incorporated

into phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) in rodent MP (83) and that rapid turnover of these phospholipids is accompanied by selective AA release following stimulation with certain factors (80). Although stimulated AA metabolite production appears to be consistent with enhanced AA release from MP phospholipid stores, direct correlation of the time course of AA release from MP phospholipids with the appearance of eicosanoid products has not been established.

Increased immunoreactive PGE and TXB_2 release has been observed from human mononuclear phagocytes (HMP) maintained in culture for 24 hr in the presence on C3b, iC3b, C3c (72), endotoxin (LPS) (80), and antigen-antibody complexes (73). These studies were unique in that highly enriched HMP were first obtained from freshly drawn blood through the use of counterflow centrifugation (CC) (74). This technique permits rapid enrichment of HMP in suspension, thus avoiding selective adherence in mixed culture , which is the most frequently used technique to isolate MP. Utilizing CC separated HMP, it was shown that 24 hr cultures exposed to C3b demonstrated decreased PI levels and increased lysophosphatidylcholine (LPC) and sphingomyelin levels when compared to control cultures (42). Although these findings support the proposed relationship between AA release from lipid stores and subsequent conversion to eicosanoid products, there is no direct evidence demonstrating release

of preesterified AA in C3b stimulated HMP and subsequent conversion of released AA to eicosanoid products. We report here experiments which examine this relationship in C3b treated HMP by comparing the time course of labelled AA release and conversion to metabolites with the release of immunoreactive eicosanoids. In addition, labelled metabolite release was examined after treating HMP with LPS.

Materials and Methods:

Materials. [^{3}H]Arachidonic acid (87 Ci/mmole), [^{3}H]TXB_2 (113 Ci/mmole), and [^{3}H]PGE_2 (165 Ci/mmole) were purchased from New England Nuclear. Phospholipid standards were obtained from Supelco. Arachidonic acid, TXB_2, PGE_2, 2-p-toluidinylnaphthylene 6-sulfonate (TNS), penicillin and streptomycin were obtained from Sigma Chemical Co. MCDB 104 was purchased from Flow Laboratories. Anti-PGE_2 antibody (generous gift of Dr. Lole) and anti-TXB_2 antibody (Seragen) were used for radioimmunoassay. Fetal calf serum (FCS) was obtained from Hyclone Laboratories. C3b was prepared by by mild tryptic cleavage of purified C3 and separated from C3a by gel filtration on Sephadex G-75 or Sephacryl S200 at pH 4.5 (72). Preparations were assessed for purity and characteristic antigenicity by immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis. Purified proteins were dialyzed against PBS, pH 7.5, and passed through 0.45μm pore filters prior to being used in culture. LPS (Salmonella typhimurium) was purchased from Calbiochem-Behring.

Cell isolation and culture. Human monocytes were isolated by counterflow centrifugation (CC) as previuosly described (74). Briefly, citrated fresh human blood, obtained by venipuncture, was separated by ficoll-Hypaque density gradient centrifugation to yield a highly enriched mononuclear cell fraction. This cell fraction was then subjected to CC during which platelet and lymphocyte elution was monitored by electronic sizing of effluent cells from CC (Coulter ZM-counter, Coulter Electronics, Hialeah, FL). Once the monocyte fraction had been sufficiently enriched, centrifugation was terminated and monocytes quickly eluted from the separating chamber. Monocytes were then pelleted in Ca-Mg free PBS with 1% autologous citrated plasma and resuspended in appropriate medium for culture inoculation.

The cells were resuspended in a known volume of MCDB 104 containing 1% FCS, 100 U/ml penicillin, and 100 mcg/ml streptomycin, and monocyte recovery determined by electronic counting. The cell suspension was then diluted to achieve a concentration of 1.0-1.3 x 10^6 cells/ml and 1 ml aliquots were placed in glass wells (18 mm diameter). For experiments evaluating labelled AA metabolism, [^{3}H]AA was added to the monocyte suspension at 0.1 μCi/ml and cells inoculated at the same density. All cultures were then incubated at 37°C for 2 hr in a humidified atmosphere of 5% CO_2 in air after which the cultures were washed 2 times to eliminate unincorporated label and/or nonadherent cells. Cultures were

then treated with media containing C3b (15 ug/ml) or LPS (10 ug/ml) vs control medium.

At 2 8 16 and 24 hr, the cell-conditioned medium (CM) was replaced with identical fresh medium and the CM centrifuged at 2500 x g for 5 min. Supernatants were then stored frozen under N_2 in sealed glass tubes until analyzed by RIA for PGE and TXB_2. In the case of labelled cultures, medium was exchanged at identical time points and 100 ul of supernatant counted to determine [^{3}H] release. The remaining supernatant was stored as described above until extraction of labelled AA metabolites.

RIA for PGE and TXB_2. Immunoreactive PGE present in the culture supernatants was determined by radioimmunoassay using ammonium sulfate precipitation of monoclonal anti-PGE_2 antibody. PGE content of culture supernatants was extrapolated from logit-log PGE_2 standard binding with each assay (detection limit of 100 pg/ml). PGE_2 stock solution was diluted in Tris buffer (10mM Tris, 140 mM NaCl. pH 7.6 with 2 mg/ml human gamma globulin) to give dilutions of 100 to 50,000 pg/ml. Anti-PGE_2 stock solution was prepared by diluting 20 µl monoclonal antibody stock in 1 ml of Tris-gamma globulin buffer. PGE content was then determined by [^{3}H]PGE_2 counts remaining in the assay supernatant after ammonium sulfate precipition. All determinations were carried out in duplicate and the average counts for each pair used to calculate PGE content. Regression coefficients

of logit-log standard curves were generally greater than 0.990.

Absorption by activated charcoal rather than immunoprecipitation was used to determine TXB_2 by RIA. All solutions were prepared in Tris buffer (10mM Tris, 140mM NaCl, pH 7.4, with 0.1 % gelatin). Anti-TXB_2 stock solution was prepared by diluting 1 vial of antibody (Seragen) in 20 ml of Tris-gelatin buffer. Generally, 10 µl of culture supernatant was assayed for TXB_2 content. TXB_2 content was determined by supernatant [^{3}H]TXB_2 counts remaining after Norit-A absorption of unbound TXB_2 according to a commercially available kit (Seragen). The TXB_2 standard curve and unknowns were calculated by logit-log transformation and linear regression as with PGE RIA.

Labelled arachidonic acid metabolite extraction and separation. AA metabolites were extracted by first adjusting the pH of culture supernatants to 3.0 with formic acid (70%) followed by two 3ml washes with ethyl acetate. The combined organic extracts were evaporated under vacuum and stored at -20°C under N_2. Prior to TLC separation of AA metabolites, AA (5µg), TXB_2 (5µg), and PGE_2 (5µg) were added to each sample and the contents dried under a stream of N_2. Individual samples were dissolved in 10 µl of chloroform, vortexed, and spotted on LHP-K plates (precoated 200 µm silica gel G, Whatman). This was followed by 3 additional washes with 5 µl of chloroform, spotting each wash

immediately below the previous application. Plates were then developed in 1 dimension with chloroform:methanol:acetic acid:H_2O (90:8:1:0.8 v/v) (98). After drying, the plates were sprayed with fluorescent indicator (1% TNS in 50 mM Tris, pH 7.4) and lipid spots visualized with UV light. AA metabolites were identified by comigration with authentic standards after which spots were scraped and counts determined.

Phospholipid extraction and separation. Phospholipids were extracted directly from glass wells using a modification of the method of Billah et al. (8). Briefly, wells were washed 4 times with 0.5 ml of chloroform:methanol:HCl (10:20:1 v/v) after which fewer than 100 cpm could be recovered from any well. The pooled organic extracts from individual wells were then brought to 4 ml with the same extraction solvent followed by the addition of 1.2 ml of 2 M KCl and 1.2 ml of chloroform. The samples were vortexed and centrifuged for 5 min at 1500 x g to facilitate phase separation. The upper phase was discarded and the lower organic phase transferred to Reacti-vials and dried under a stream of N_2. The extraction efficiency of this method was found to be virtually identical to the neutral pH-high salt extraction procedure of Garbus et al. (24).

Phospholipids were separated by 1 dimensional TLC employing the following solvent system, chloroform:ethanol:triethylamine:H_2O (30:34:35:8 v/v) (95). One dimensional

phospholipid separations were used as a preliminary means to identify alterations in [^{3}H]AA content of phospholipids. For this separation, lipids were dissolved in 10 μl of chloroform, vortexed, and spotted on LHP-K plates (precoated 200 μm, silica gel G, Whatman). The sample vial was then washed 3 times with 5 μl of chloroform, spotting each wash immediately below the previous application. The plates were then developed and lipids visualized with 1% TNS (see above). Individual lipids were identified by comigration with authentic standards and counts determined by scraping spots.

Results:

Release of radiolabel in culture medium by HMP prelabelled with [^{3}H]AA. Figure 1 shows the effect of C3b on [^{3}H] appearance in medium equilibrated with HMP monolayers attached to both glass and plastic culture wells (16 mm diameter, Falcon). HMP were labelled with [^{3}H]AA for 2 hr and washed free of any unincorporated label prior to stimulation. Plastic and glass cultureware were compared because previous investigations of C3b effects on HMP eicosanoid release have utilized cells attached to plastic dishes only (72,80,73). The use of glass wells facilitated direct organic extraction of cellular phospholipids which was not possible with plastic cultureware. As can be seen, comparable [^{3}H] release was observed for HMP cultures adherent to glass and plastic. It should be emphasized that

Figure 1. Comparison of time dependent [^{3}H] release from control or C3b treated HMP cells attached to glass or plastic culture wells. Culture supernatants were obtained by centrifuging HMP conditioned medium at 2500 x g for 5 min. Each determination represents the mean ± S.E. for 4 trials. The release values are for plastic wells with either control medium () or 15 ug/ml C3b () and for glass wells with either control medium () or 15 ug/ml C3b (). All subsequent experiments utilize HMP attached to glass dishes only.

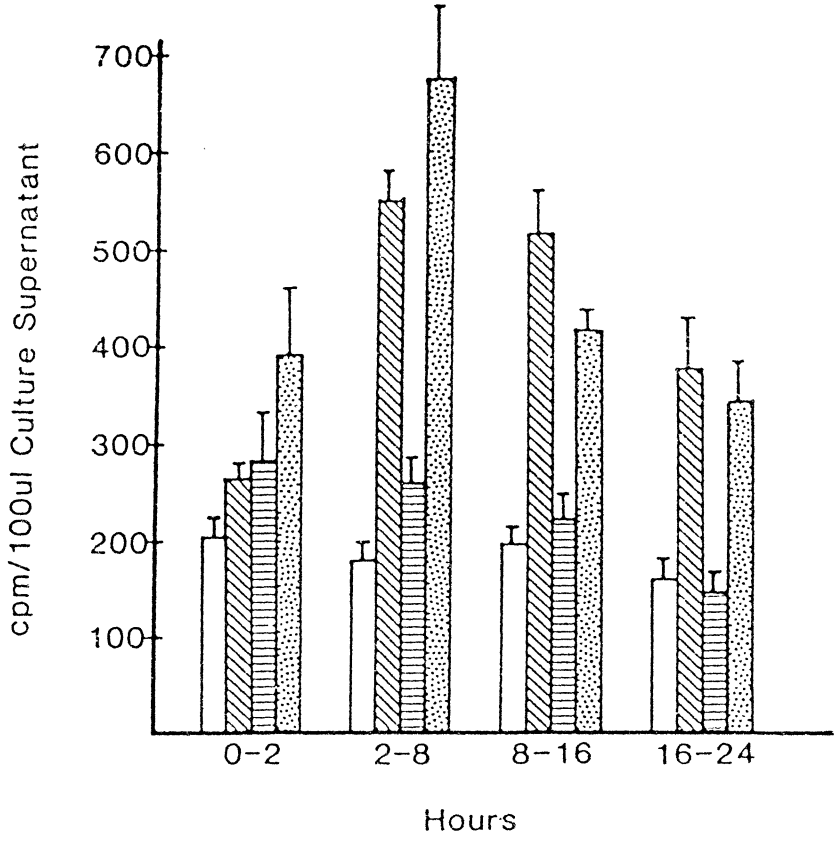
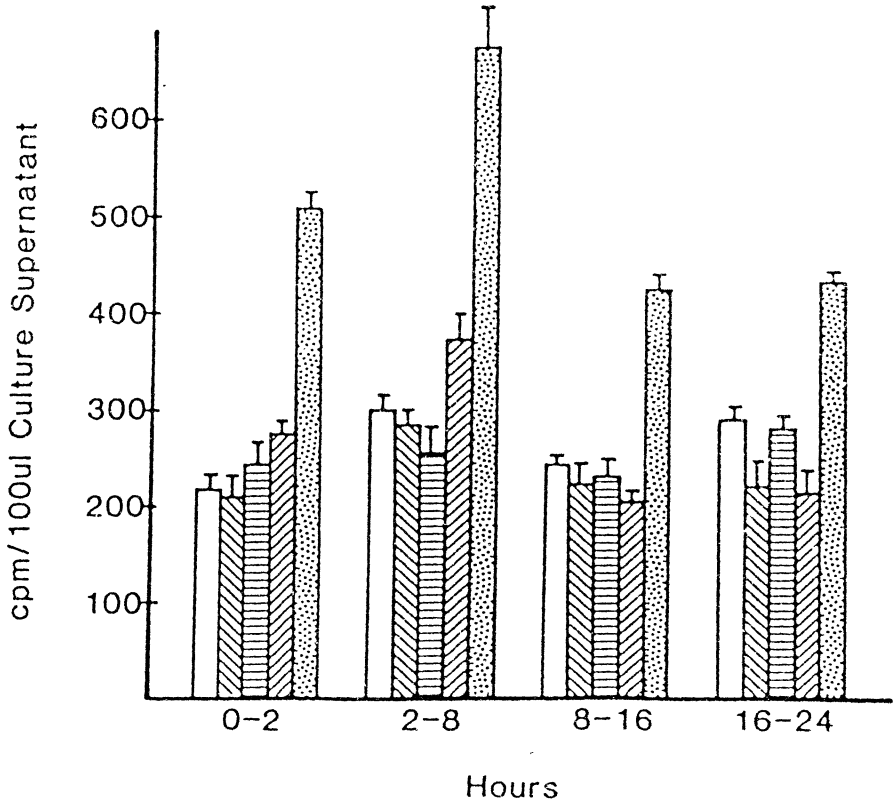

700
600
500
400
300
200
100
cpm/100ul Culture Supernatant
0-2
2-8
8-16
16-24
Hours

Figure 2. C3b dose dependent [^{3}H] release from HMP in culture. Control () release represents the mean ± S.E. for 8 trials. For all other determinations, n=4. C3b concentrations were as follows: 0.015 μg/ml (), 0.15 μg/ml (), 1.5 μg/ml (), and 15 μg/ml ().


cpm/100ul Culture Supernatant
600
500
400
300
200
100
0-2
2-8
8-16
16-24
Hours

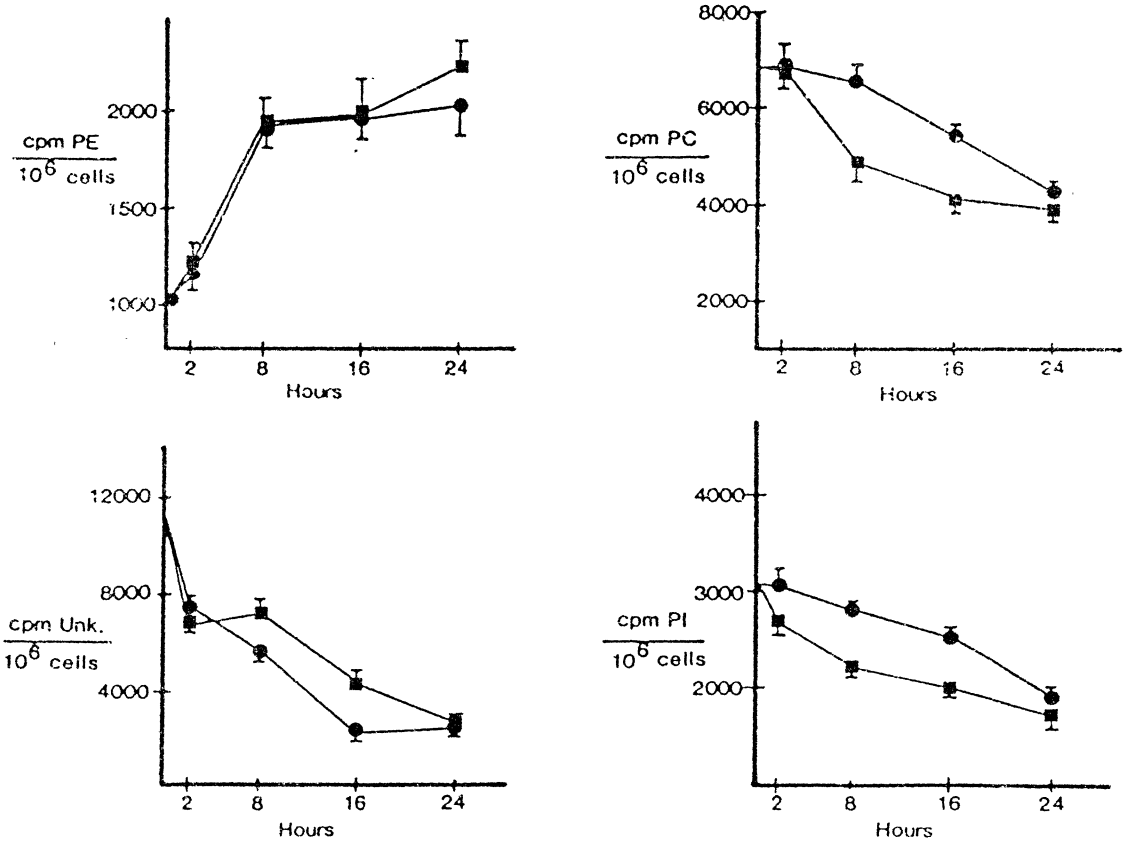
doses of C3b also did not stimulate release of [^{3}H] over control cultures.

Effects of C3b on [^{3}H]-phospholipid content. Figure 3 demonstrates the time dependent alterations in [^{3}H]AA label within HMP lipid classes with and without C3b stimulation. As shown, C3b reduced the label within PI and PC without affecting labelling of PE. C3b increased the rate of [^{3}H] label clearance from PC and PI for up to 8 hr of stimulation after which label clearance was reduced to that seen in control cultures. It was also interesting that lipids migrating at the solvent front (Unk.), presumably neutral lipids, increased [^{3}H] content between 2 and 8 hr after C3b stimulation. This increase coincided with the maximal decrease in PI and PC labelling following C3b stimulation.

In these experiments, lipids were separated by 1 dimensional TLC (see methods) as a preliminary evaluation of of alterations in label distribution. Definitive studies are now in progress to confirm these results by 2 dimensional TLC and to extend this study to identify phospholipid turnover events in association with alterations in [^{3}H] label distribution following C3b treatment.

The data in Figure 3 also demonstrate the time dependent redistribution of label between lipid classes within control or C3b stimulated HMP. These results indicate that 2 hr exposure to exogenous [^{3}H]AA resulted in label incorporation primarily into PI, PC, and presumably neutral

Figure 3. Effect of C3b on [^{3}H] label content of selected HMP lipids. Each point represents the mean ± S.E. for 3 trials. With the exception of lipids migrating at the solvent front (Unk.), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI) were identified by comigration with authentic standards. Data points represent control medium (●) and 15 μg/ml C3b (■).


cpm PE
10^6 cells
2000
1500
1000
2
8
16
24
Hours
cpm PC
10^6 cells
8000
6000
4000
2000
2
8
16
24
Hours
cpm Unk.
10^6 cells
12000
8000
4000
2
8
16
24
Hours
cpm PI
10^6 cells
4000
3000
2000
2
8
16
24
Hours

lipids. Subsequent to this initial uptake, and in the absence of exogenous [^{3}H]AA, label was redistributed from PI, PC, and neutral lipids into PE and other lipids with increasing time in culture. This phenomenon was also suggested by the label distribution observed in HMP lipids following 2 or 26 hour exposure to identical amounts of [^{3}H]AA. PI and PC demonstrated only modest increases (approximately 25%) in [^{3}H] content with an additional 24 hr exposure to [^{3}H]AA whereas PE and PS demonstrated greater than 4-fold increased labelling between 2 and 24 hr in culture (data not shown).

C3b and LPS stimulated release of PGE and TXB_2 determined by RIA. Figure 4 shows the effect of C3b (15 μg/ml) on TXB_2 and PGE release from HMP cultures derived from 2 donors. As with [^{3}H] release from HMP cultures, the release of AA metabolites was cumulative over the indicated time interval. The time course of TXB_2 release was considerably different from PGE release. When metabolite release was expressed in ng/hr, TXB_2 release demonstrated a maximal rate during the 2-8 hr time interval whereas PGE release was maximal after 16 hr of C3b stimulation.

Figure 5 demonstrates the effect of LPS and C3b on PGE release from HMP cultures prepared from the same donor. The release of PGE from HMP control cultures in this preparation was appreciable, in contrast to the control release in Figure 5. Conversely, C3b-stimulated release of PGE was

Figure 4. Time course of TXB_2 and PGE release from HMP following stimulation with C3b (15 μg/ml). Each value represents the mean ± S.E. for 6 trials for TXB_2 () and PGE (). Values represent the average response for HMP isolated from 2 donors. TXB_2 release from control cultures did not exceed 1 ng/ml and PGE release from controls did not exceed 0.1 ng/ml.

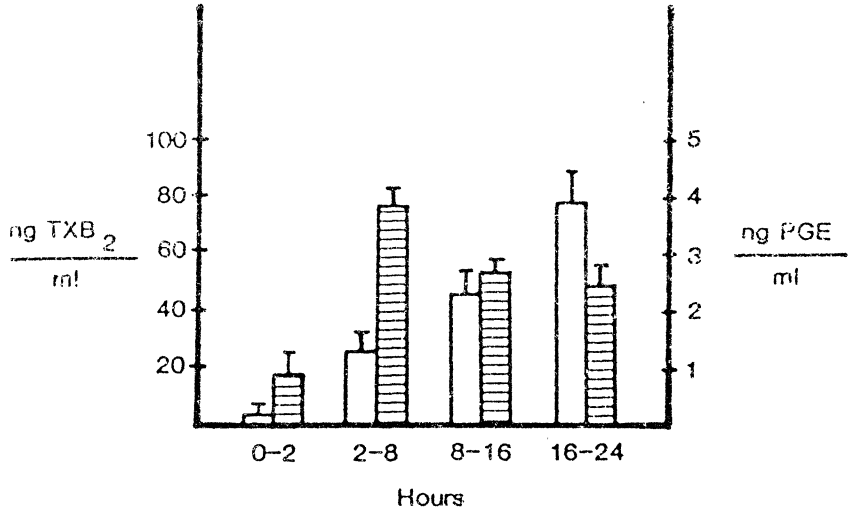
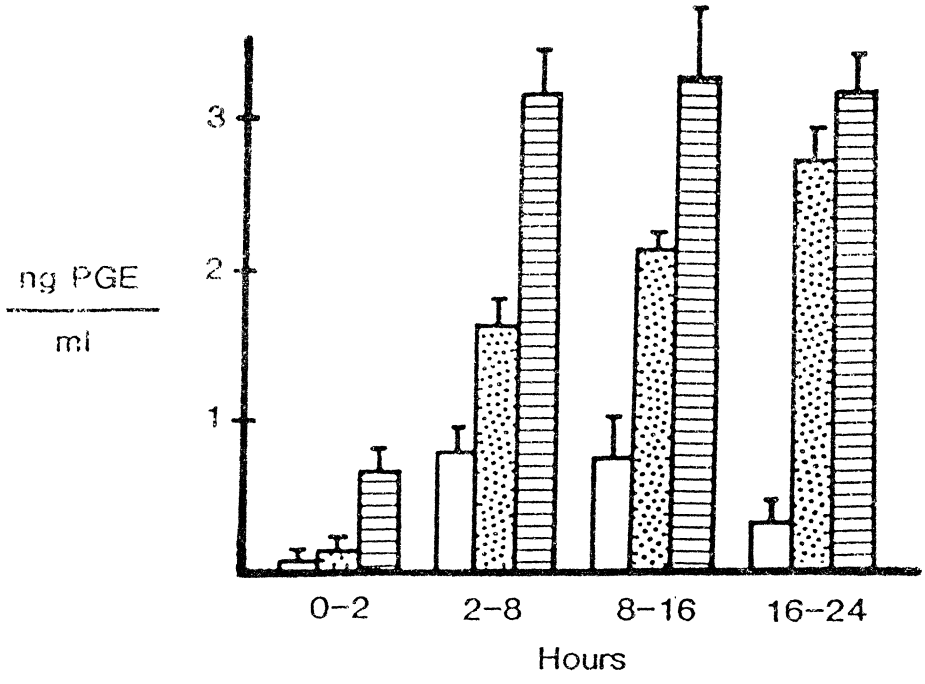

ng TXB 2
ml
100
80
60
40
20
5
4
3
2
1
ng PGE
ml
0–2
2–8
8–16
16–24
Hours

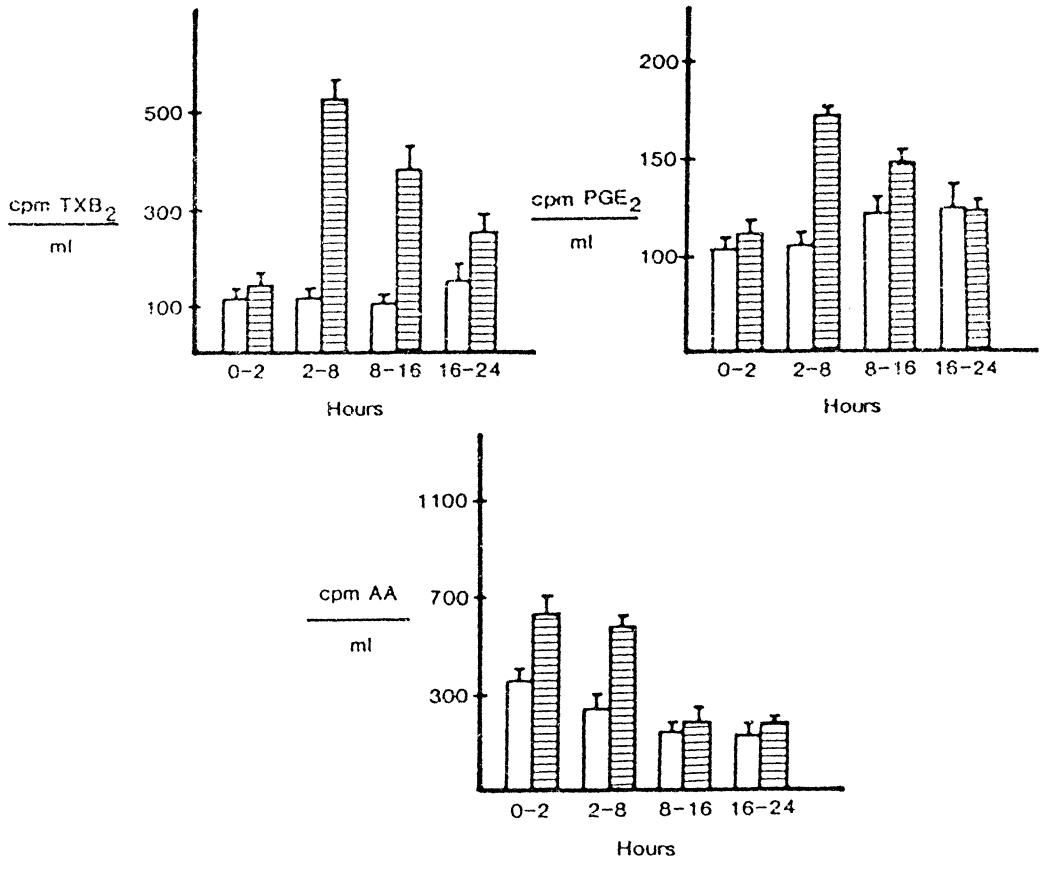
Figure 5. PGE release from HMP cultures stimulated with C3b or LPS. Each point represents the mean ± S.E. for 6 trials. HMP were isolated from the same donor. The release values are for control medium (□), 10 μg/ml LPS (▤), and 15 μg/ml C3b (▩).


ng PGE
ml
3
2
1
0–2
2–8
8–16
16–24
Hours

comparable to that observed in Figure 4. A previous report has also noted considerable variation in the stimulated and control levels of PGE and TXB_2 released by cells from different donors (72). Cultures stimulated with LPS (10 μg/ml) demonstrate maximal release of PGE during the 2-8 hr time interval, considerably earlier than was observed with C3b. However, C3b treated cultures appeared to achieve the same rate of PGE release as the LPS-treated cultures by 24 hr. No TXB_2 RIA was carried out on these samples. Whether C3b and LPS mediate different effects on HMP eicosanoid production will be the subject of future studies.

Determination of labelled AA, TXB_2, and PGE_2 levels in HMP culture supernatants. Figure 6 shows the time dependence of labelled AA, ,TXB_2, and PGE_2 release from HMP cultures following stimulation with C3b. As in previous experiments, the metabolite release was cumulative only for the indicated time interval. Stimulated release of [^{3}H]AA was observed for up to 8 hr after C3b treatment followed by a return to control release levels. In contrast, [^{3}H]TXB_2 and [^{3}H]PGE_2 release was not stimulated during the initial 2 hr interval after C3b treatment but was maximally stimulated during the 2-8 hr interval. In addition, continued stimulation of labelled TXB_2 and PGE_2 release was observed for the 8-16 hr interval. Also noteworthy was the similar pattern of labelled metabolite release between TXB_2 and PGE_2 whereas TXB_2 and PGE release determined by RIA were found to be

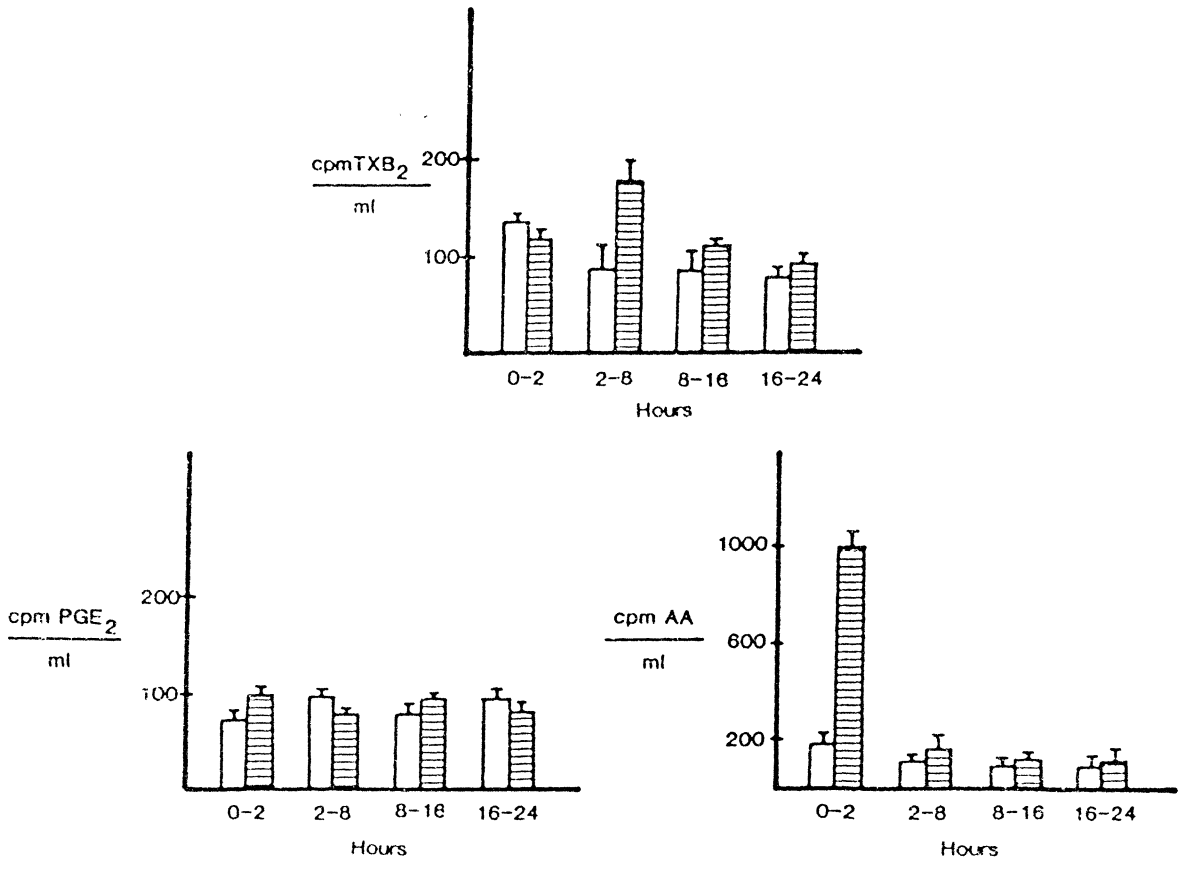
Figure 6. Effect of C3b on [^{3}H] labelled AA, TXB_2, and PGE_2 release from HMP. Each value reprasents the mean $\pm$ S.E. for 3 trials. The values represent labelled metabolite release for control medium () and 15 μg/ml C3b ().


cpm TXB_2
ml
500
300
100
0-2
2-8
8-16
16-24
Hours
cpm PGE_2
ml
200
150
100
0-2
2-8
8-16
16-24
Hours
cpm AA
ml
1100
700
300
0-2
2-8
8-16
16-24
Hours

considerably different (see Figure 4). In addition, the significant C3b effects on labelled metabolite release have been corroborated by HPLC analysis of conditioned medium extracts from parallel experiments. A complete report of the HPLC results is in preparation.

LPS stimulation of [^{3}H]AA labelled HMP in culture gave a similar pattern of labelled metabolite release. Figure 7 shows the time dependent release of labelled AA, TXB_2, and PGE_2 from HMP cultures stimulated with LPS (10 μg/ml). HMP used in this experiment were the same as those used in Figure 5. As shown, labelled AA release was stimulated during the 0-2 hr interval of LPS stimulation but returned to control values thereafter. Labelled TXB_2 and PGE_2 release were not stimulated for up to 2 hr. Labelled TXB_2 release was maximally stimulated during the 2-8 hr interval and subsequently returned to control levels. In contrast to C3b-stimulated metabolite release, significant release of labelled PGE_2 was not observed after stimulation with LPS.

Figure 7. Effect of LPS on [^{3}H] labelled AA, TXB_2, and PGE_2 release from HMP. Each value represents the mean ± S.E. for 3 trials. Metabolite release was determined for control medium () and 10 μg/ml LPS ().


cpmTXB2
ml
200
100
0-2
2-8
8-16
16-24
Hours
cpm PGE2
ml
200
100
0-2
2-8
8-16
16-24
Hours
cpm AA
ml
1000
600
200
0-2
2-8
8-16
16-24
Hours

Discussion:

The time interval assessment of radioisotope release from [^{3}H]AA labelled HMP served to identify intervals of varying metabolite release. Although the time intervals described in this report were empirically developed from experimental trials, they were intended to address the previously reported differential rates of PGE and TXB_2 release observed from C3b stimulated HMP (80). The desirability of breaking the stimulation period into several intervals relates to the nature of the metabolite release. As has been observed in several reports, cumulative AA metabolite release from stimulated macrophages and monocytes typically demonstrates a rapid initial release which tends to plateau with increasing time of stimulation. However, calculation of the average rates of metabolite production becomes less precise when approaching the plateau region of cumulative metabolite release. Yet, this is the time period where differential TXB_2 and PGE release has been observed from C3b-stimulated HMP (80). By exchanging medium at discrete intervals, it was possible to obtain a more sensitive measure of average rates of metabolite release during the attenuated response period. This approach assumes that metabolite production during the early time periods does not alter subsequent metabolic events. However, as will be shown, comparison of our results with previous findings demonstrates that our experimental design does not

discernably affect the biological response previously characterized for HMP.

According to this construct, it was possible to identify four intervals demonstrating different average rates of radioisotope release following C3b stimulation of HMP. These intervals were as follows: 0-2 hr interval of increasing label release, 2-8 hr interval of maximal label release, and 8-16 and 16-24 hr intervals of decreasing label release. With some experiments, the 0-2 hr and 2-8 hr intervals demonstrated approximately the same average responses, when expressed as cpm released/hr. LPS stimulation of [^{3}H]AA labelled HMP produced a very similar pattern of radioisotope release to that observed in C3b stimulated HMP. However, C3b did not stimulate [^{3}H] release in this same HMP cell preparation. Previously, it was shown that HMP prepared from fresh blood demonstrate a time-dependent loss of C3b-stimulated PGE and TXB_2 release that was not correlated with a decrease in C3b receptor-bearing cell numbers (79). The loss of C3b responsiveness observed with aged buffy coat HMP may represent the same process since these cells were obtained approximately 24 hr prior to HMP separation and culture. Future studies will address this question.

[^{3}H]AA incorporation into HMP phospholipids was comparable to that observed previously in rodent macrophages (83,64). [^{3}H]-AA was incorporated primarily into PC after 2

hr exposure whereas exposure to [^{3}H]AA for 26 hr resulted in much greater percentage of label incorporated into PE (data not shown). The time-dependent redistribution of label in HMP observed as a net loss of label from PC and PI and a net increase of label in PE, has also been observed in mouse macrophages (83). C3b-stimulated [^{3}H] label release from PC and PI without affecting PE label content is in general agreement with previous reports demonstrating zymosan and f-met-leu-phe stimulated label release from PI and PC in rodent macrophages prelabelled with [^{3}H]AA (106,64). Also noteworthy is the recent report demonstrating decreased PI and increasing LPC and sphingomyelin levels in HMP treated with C3b (42). Taken together with the present findings, it appears that decreased [^{3}H] label in PI may reflect increased breakdown of PI whereas decreased label in PC may reflect increased turnover of PC without a net breakdown of this phospholipid. Experiments are now in progress to evaluate these possibilities. The observed transfer of [^{3}H] label into lipids migrating at the solvent front suggests that neutral lipids accumulate label following C3b stimulation of HMP. This is consistent with the report of Bonney et al. (64), where a net transfer of [^{3}H] label into triglycerides was observed with zymosan stimulation of [^{3}H]AA-labelled mouse macrophages. These results therefore indicate that medium exchanges at the described intervals do not adversely affect [^{3}H] label release from HMP lipid

pools. C3b-stimulated release of PGE and TXB_2 from HMP in this study is in general agreement with previous reports. Schenkein and Rutherford demonstrated that maximal stimulation of PGE release occured 20-24 hr after C3b treatment of HMP with little or no release during the first 4 hr of stimulation (80). In contrast, TXB_2 release was stimulated by 1 hr and appeared to be approximately linear over the 24 hr exposure to C3b (80). These results were established for HMP treated with C3b at the time of culture inoculation and for culture medium containing 1% FCS. In agreement with the results of Schenkein et al. (72,80), we observed maximal PGE release during the 16-24 hr interval after C3b stimulation. Our results differ only in that we observed a time dependent increase in the rate of PGE release which was detectable during the initial 2 hr exposure to C3b.

The apparent differences in the time course of TXB_2 and PGE release from stimulated HMP suggest either selective regulation of eicosanoid metabolism or that immunoreactive PGE is predominantly PGE_1. The latter possibility is unlikely since the fatty acid composition of human monocytes has been shown to include 20% arachidonic acid (87) with no significant levels of linolenic acid (87,64), the precursor fatty acid for dihomo-g-linolenic acid (DHL). DHL is converted to PGE_1 by way of cyclo-oxygenase. Mouse peritoneal macrophages have been shown to synthesize PGE_1

when prelabelled for 4 hr with [^{3}H]eicosatrienoic acid (DHL) and stimulated with zymosan (11). However, parallel experiments using [^{3}H]AA as label demonstrated PGE_2 as the predominant PGE metabolite released from zymosan treated mouse MP (29). In our experiments, the possibility exists that fetal calf serum provided significant amounts of linolenic acid and/or DHL which was converted to PGE_1 by stimulated HMP. Deletion of fetal calf serum from HMP culture medium would therefore be expected to reduce PGE release without affecting TXB_2. However, PGE and TXB_2 release from C3b stimulated HMP were equally diminished (approximately 30%) with the removal of fetal calf serum from culture medium (80). Because PGE release was not selectively and substantially diminished under these conditions, these results suggest that fetal calf serum is not promoting PGE_1 production in stimulated HMP.

The time course of immunoprecipitable TXB_2 and PGE release from C3b-stimulated HMP suggests regulation of AA metabolism so that TXB_2 is produced earlier than PGE. This could result from enzyme regulation which preferentially catalyzes AA conversion to TXB_2 during early time periods followed by a subsequent increase in the conversion of AA to PGE_2. This possibility was examined by measuring the time course of radiolabelled AA conversion to TXB_2 and PGE_2 in C3b stimulated HMP. It was found that labelled and immunoprecipitable TXB_2 release were very similar whereas

the release of labelled PGE did not parallel the release of immunoprecipitable PGE. Assuming that immunoprecipitable PGE represents primarily PGE_2, these results indicate that the specific activity of PGE_2, expressed in cpm/ng (see Figures 4 and 6), declined over the 24 hour stimulation period while the specific activity of TXB_2 remained virtually unchanged. Because cyclo-oxygenase conversion of AA to PGG_2 serves as the single common pathway for the production of TXB_2 and PGE_2, these results are inconsistent with a time dependent shift in enzymatic production from TXB_2 initially to PGE_2 subsequently. Instead, these results suggest that C3b and LPS stimulated HMP synthesize TXB_2 and PGE_2 from independent cell sources of AA. The LPS effects were included in this manuscript because they demonstrated the same basic pattern of labelled and immunoprecipitable metabolite release as was demonstrated by C3b. That independent metabolism of AA can occur in C3b- and LPS-stimulated HMP is also suggested by the early release of labelled AA into culture supernatants without the appearance of labelled metabolites. In addition, the time course of label disappearance from phospholipids of C3b-treated HMP correlated only with the appearance of labelled TXB_2. A similar increase in label release was not observed during the period of maximal PGE release from C3b-stimulated HMP. It is possible that longer labelling periods with [^{3}H]AA are required to label the requisite lipid pools in HMP to observe parallel release of labelled and

immunoprecipitable PGE_2.

The possibility of independent AA metabolite production has been suggested in other reports. Using elicited and resident mouse peritoneal macrophages, Scott et al. (82) demonstrated that AA incorporation into lipid pools can be independent of synthesis of AA metabolites. Goldyne and Stobo extended this observation by demonstrating that exogenous [^{14}C]AA previously released by T cells could be converted to TXB_2 in human macrophages (28). However, no labelled PGE_2 was detected in the same culture medium even in the presence of elevated immunoprecipitable PGE release. Because PGE levels were 15-20% of TXB_2 levels by RIA, it was suspected that [^{14}C]PGE_2 counts would be indistinguishable from background counts in the chromatographic separation. Our studies suggest that Goldyne and Stobo did not observe labelled PGE_2 release because [^{14}C]AA had not equilibrated into the requisite metabolic compartment for conversion to PGE_2. Studies are now in progress to examine the AA lipid pools in HMP which give rise to PGE_2 and TXB_2.

Thesis Discussion:

The previously described studies compare arachidonic acid (AA) metabolism in two different cell types following treatment with respective soluble agents known to elicit biological responses. The osteoblast-like clonal cells, ROS 17/2·8, demonstrate little if any capacity to alter phospholipid-AA metabolism when stimulated with PTH. Conversely, human mononuclear phagocytes (HMP) in vitro exhibit pronounced alterations in phospholipid AA metabolism when stimulated with C3b. The variability in responsiveness demonstrated in the present study is not inconsistent with responses noted in other cells. Some hormones have been shown to be without effect on phospholipid or AA metabolism in their respective target tissues, i.e., glucagon. Conversely, very pronounced alterations in phospholipid metabolism and increased AA release have been observed in hormonally or chemically stimulated cells, particularly white blood cells. The variation in cell responses observed in the present study were therefore not entirely unexpected.

The lack of PTH effects on ROS cell phospholipid-AA metabolism was in contrast to PTH stimulation of cAMP levels and inhibition of alkaline phosphatase activity. These findings indicate that ROS cells express osteoblast-like responsiveness to PTH when grown in microcarrier culture. The lack of PTH effects on phospholipid-AA metabolism in ROS cells grown in microcarrier culture contrasted with the

previously reported effects of PTH on phospholipid metabolism in kidney (5). In addition to physiological regulation of Ca and PO_4 release from bone, PTH acts to regulate Ca reabsorption and PO_4 clearance from kidney; thus, kidney is another important target tissue for PTH action. Stimulation of dog kidney with PTH has been shown to decrease PI levels, increase labelled PO_4 uptake into PI and PA, and to increase prostaglandin synthesis (5). Although ROS cells and kidney demonstrate two extremes of PTH responsiveness with respect to phospholipid-AA metabolism, variation in tissue responsiveness of this magnitude has been observed with other chemical agents. For example, β-adrenergic stimulation of cerebral cortex is without effect on phospholipid metabolism whereas treatment of other tissues, including vas differens, with β-adrenergic agents can produce significant alterations in phospholipid turnover (60). The effects of PTH on ROS cells observed in the present study are therefore not unique.

The ability of ROS 17/2·8 cells to produce PGE_2 in culture is a perplexing issue. Particularly noteworthy is the capacity of PGE_2 to stimulate osteoclastic-mediated bone resorption *in vitro* and *in vivo* (44,78). However, PTH-stimulated osteoclastic resorption in organ culture has been shown to be independent of prostaglandin release (51,66). The observed release of PGE_2 from ROS 17/2·8 cells indicates the presence of cyclo-oxygenase enzyme activity. However, it

does not explain the source of AA for conversion to PGE_2. It is possible that AA present in the culture medium served as substrate for the production of PGE_2. This possibility appears likely since labelled PGE_2 release from ROS 17/2·8 cells was greatest during the initial 2 hour period of exposure to [^{14}C]AA (71). Presumably, the rate of PGE_2 release diminished due to the uptake of exogenous AA into ROS lipids thereby reducing labelled AA availability to cyclo-oxygenase enzyme systems. It is also possible that [^{14}H]AA may transiently label a lipid/phospholipid in ROS cells which turns over rapidly and provides free AA for conversion to PGE_2. However, AA labeling of ROS phospholipids has not confirmed the presence of transient phospholipid labeling. In summary, it is uncertain how ROS cells regulate AA metabolism such that basal production of PGE_2 occurs at a significant rate.

The microcarrier culture system used in the present studies was unique in that cells were grown in monolayer culture attached to very small dextran beads maintained in suspension through gentle stirring. ROS cells demonstrated comparable PTH responsivenness in microcarrier culture as was previously observed for cells grown on conventional plasticware. PTH responses assayed were stimulation of cAMP and inhibition of alkaline phosphatase. However, there are mechanical agitation properties associated with microcarrier culture which might affect PGE_2 release. ROS cells have been

shown to increase PGE_2 release when subjected to mechanical stretching (107), as has been observed in many other cell types including fibroblasts (62). The possibility that microcarrier culture induced alterations in AA metabolism was examined by comparing [^{3}H] release from [^{3}H]AA labelled ROS cells maintained in microcarrier and stationary culture. It was found that label release was identical for both culture systems. In addition, transfer of cell samples from microcarrier culture to stationary culture was not associated with any redistribution of [^{3}H]AA within phospholipid classes. These results suggest that microcarrier culture does not significantly affect endogenous AA metabolism in ROS 17/2·8 cells.

The Ca^{++} ionophore, A-23187, was the only agent in our study which stimulated labelled AA release from ROS cells. A-23187 facilitates Ca^{++} influx into cells which stimulates phospholipase A_2 activity and AA release from phospholipids. A 23187 has been shown to be ineffective in other cell systems in the absence of extracellular Ca^{++} (55). In addition, the Ca^{++} binding protein, calmodulin, is also implicated in A-23187 activation of phospholipase A_2, since the calmodulin inhibitor, trifluoroperozine (TFP), will block A-23187 effects on phospholipase A_2 in platelets (104). PTH was expected to produce a response similar to A-23187 since PTH has been shown to stimulate ^{45}Ca uptake into osteoblast enriched populations of freshly isolated bone

cells (18,19). Enhanced uptake of ^{45}Ca uptake is generally accepted as a measure of increased uptake of Ca^{++} by cells. Since ROS 17/2·8 cells demonstrate comparable PTH responses to osteoblast-enriched populations of bone cells, it was anticipated that the ROS cell line would serve as a good model to test this hypothesis. Because a significant dose of PTH had a virtually insignificant effect on AA release from ROS cells relative to A-23187, it appears that PTH does not elicit effects on ROS cells through a mechanism comparable to A-23187.

The inability of PTH to stimulate endogenous AA metabolism in ROS 17/2·8 cells does not necessarily indicate that hormonally stimulated osteoblasts in vivo or in primary culture respond in a similar manner. Recent evidence has shown PTH stimulation of PGE production in osteoblast-enriched cells (53). In addition, it is equally possible that bone cells other than osteoblasts respond to PTH with an increase in endogenous AA metabolism. It is not established which cell types are responding under these experimental conditions and the mechanisms regulating AA metabolism. However, the inability to isolate pure populations of individual bone cell types from intact bone makes these findings difficult to resolve. Future research in this area will be limited until relatively pure populations of individual bone cell types can be conveniently isolated.

The characterization of AA metabolism in HMP was an extension of previous work by Rutherford and Schenkein. Together with other workers, these investigators demonstrated enhanced 24 hour release of PGE and TXB_2 from C3b treated HMP. C3b treatment was also shown to decrease HMP PI levels and increase sphingomyelin and lysophosphatidylcholine levels over the same treatment period. This preliminary work suggested that phospholipid-AA metabolism might be important in regulating PGE and TXB_2 release from HMP. The present study therefore examined this relationship by comparing the time course of [^{3}H]AA release from HMP phospholipids with the appearance of labelled PGE_2 and TXB_2.

In our view, the most exciting aspect of the monocyte studies was the differential pattern of eicosanoid release following stimulation with C3b or LPS. Labelled AA release occured within 2 hours of C3b or LPS stimulation without significant conversion to labelled metabolites. Conversely, the delay in labelled TXB_2 and PGE_2 release following C3b treatment was not associated with increased AA release during later time intervals. Lastly, the release of immunoreactive and labelled TXB_2 were very similar in C3b treated cells whereas the time course of immunoreactive and labelled PGE release were considerably different for the same cell samples. These results suggest a more complicated metabolic relationship between AA availability and

prostanoid production than can be explained by enzyme regulation alone.

The delay in TXB_2 and PGE_2 release from stimulated HMP appears to be unrelated to feedback regulation of AA metabolite release. Although metabolite release was determined in HMP-conditioned medium exchanged at specific time points, this method of sequential medium harvesting did not appear to significantly alter the cumulative release of immunoreactive TXB_2 or PGE. Subsequent work in our laboratory has shown that assessment of metabolite production at intervals is comparable to cumulative metabolite release over 24 hr. The sequential pattern of AA metabolite release from HMP is very interesting because it may represent an additional aspect to monocyte regulation of inflammation and tissue injury not addressed previously. These results could have considerable importance to clinical conditions which involve the release of prostanoids from monocytes.

The sequential pattern of metabolite release is also interesting with respect to mechanisms of C3b and LPS action on HMP. Human monocytes have been shown to possess cell membrane receptors for the complement cleavage products, C3b and iC3b. Without rendering a detailed explanation of the complement system (for which there are many excellent reviews), C3b is a product of C3 convertase cleavage of C3 which can be activated by either the classical or alternate

complement pathways. The iC3b fragment is the product of C3b cleavage by C3b inactivator but may also result from proteolytic activity. Monocytes have been shown to synthesize most complement proteins, including C3 as well as C3b inactivator (98). The mechanism by which LPS stimulates HMP eicosanoid production is unknown but could involve C3 cleavage on HMP membranes since LPS is a potent stimulator of the alternate complement pathway. LPS generated C3 fragments could then act through cell membrane receptors in stimulating eicosanoid release. The essential point to be made is that C3b or LPS stimulate very similar patterns of AA metabolite release from HMP. In addition, the increased metabolite release occurs after a delay of at least 2 hours after factor treatment. The release of labelled AA during the initial 2 hour treatment period probably represents plasma membrane phospholipid alterations more directly associated with C3b or LPS membrane receptor interaction or LPS treatment. On the other hand, the delay in peak release of TXB_2 and PGE after C3b or LPS treatment suggests that perhaps protein synthesis and/or RNA synthesis must take place for stimulation of metabolite production. Because the time between factor treatment and metabolite release is considerable and unusual, this cell system may provide insight into additional cellular mechanisms regulating AA metabolism.

As described in Chapter II, the differentiation of

immunoreactive from labelled PGE release suggests independent metabolism of this eicosanoid from TXB_2. Ultimately, it will be necessary to establish different [^{3}H]AA labelling conditions which demonstrate labelled PGE_2 release in parallel with immunoprecipitable PGE. It is presumed that these conditions will be different from those already established for parallel immunoprecipitable and labelled TXB_2 release. The first approach to this question will be to use longer labelling periods with [^{3}H]AA prior to stimulating with LPS or C3b. Longer labelling periods should incorporate more [^{3}H]AA into cellular lipid pools as well as allow for greater redistribution of AA into more slowly equilibrating lipid pools. The previous results suggest that most PGE can only be derived from a lipid pool which labels very slowly in the presence of exogenous [^{3}H]AA but which contains or is provided with significant amounts of unlabelled AA under stimulated conditions. By exposing cells to [^{3}H]AA for longer periods, it should be possible incorporate sufficient label into these sequestered lipid pools to measure [^{3}H]PGE_2 release which parallels immunoprecipitable PGE release.

Another approach to this problem is to determine the time course of AA loss from lipid classes in HMP following stimulation with C3b or LPS. This approach is less sensitive than the previously described labeling studies because of the complex lipid manipulations and because of the

relatively low levels of AA held within certain lipid classes in HMP. However, this approach should also provide insight into the lipids which are contributing to PGE release as well as TXB_2 and AA release.

The preliminary determinations of C3b-stimulated [^{3}H]AA release from HMP phospholipids indicate that AA is released principally from PI and PC. Further examination of C3b effects on HMP phospholipid labeling has been delayed so that a more extensive characterization of HMP phospholipids can be carried out. Recent data have demonstrated that rabbit and guinea pig macrophages possess significant levels of alkenyl-acyl and alkyl-acyl choline phosphoglyceride (CPG) and ethanolamine phosphatidylglyceride (EPG) (89,90). Alkenyl-acyl and alkyl-acyl phospholipids are distinguished from diacyl lipids in that the chemical moiety linked to the 1 position of glycerol forms an ether or an alkyl bond. Also noteworthy is that alkenyl and alkyl phospholipids of rabbit and guinea pig macrophages contain significant levels of AA (90) and that alkyl-acyl CPG may serve as a precursor molecule in the synthesis of platelet activating factor (PAF). PAF has been shown to stimulate PGE and TXB_2 release from peritoneal macrophages.

Alkyl-acyl and ether-acyl phospholipids cannot be discriminated from diacyl phospholipids by conventional 1 and 2 dimensional thin layer chromatography. However, methods are now available to conveniently characterize all

three classes of these phospholipids (94). Because AA release from PC is stimulated by C3b treatment of HMP, it is important to determine whether this release is selective for a particular class of choline phosphoglyceride. Experiments are therefore planned to first determine whether HMP contain significant levels of alkenyl-acyl or ether-acyl phospholipids and subsequently examine whether C3b or LPS can stimulate AA release.

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